INVOLVEMENT OF PFKFB3/IPFK2 IN BENEFICIAL EFFECTS OF METFORMIN ON 3T3-L1 ADIPOCYTES AND TEMPORAL EFFECTS OF PIOGLITAZONE ON ADIPOCYTES

A Thesis

by

TING QI

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ABSTRACT

Metformin, as one of the first-line clinical anti-diabetic oral drugs for T2DM, has been shown to have beneficial effects including decreasing hepatic glucose production (HGP), improving insulin sensitivity, and suppressing inflammation, primarily in the liver though AMPK pathway. However, the effect of metformin on adipose tissue and adipocytes has not been fully investigated, as well as the role of PFKFB3/iPFK2, the gene that was shown to be protective against insulin resistance and inflammatory response in adipose tissue caused by overnutrition, in beneficial effects of metformin.

In this study, the involvement of PFKFB3/iPFK2 in metformin actions is investigated using 3T3-L1 adipocytes, on the aspect of anti-inflammatory effect and improvement in insulin sensitivity. In PFKFB3/iPFK2-Control (iPFK2-Ctrl) cells, metformin treatment was shown to have significant insulin-sensitizing effect, evidenced by an increase in p-Akt/Akt signaling, and some effect on anti-inflammatory response. In contrast, insulin sensitivity was not improved, but impaired under metformin treatment on PFKFB3/iPFK2-knockdown (iPFK2-KD) adipocytes. Also, metformin did not exhibit anti-inflammatory response in iPFK2-KD cells. Meanwhile, the phosphorylation of AMPK on control cells was increased with metformin treatment, but did not alter in iPFK2-KD cells. These results supported that metformin treatment on 3T3-L1 adipocytes could increase insulin sensitivity and possibly ameliorate inflammatory response, and a disruption of PFKFB3/iPFK2 in adipocytes impairs the

insulin-sensitizing and anti-inflammatory effect of metformin, possibly through the AMPK pathway.

Other than drugs, circadian rhythm also takes an important role in regulation of adipocyte physiology. Therefore, it is pharmacologically meaningful to investigate the timing of drug delivery and the potential differential outcomes. In this study, 3T3-L1 adipocytes displayed an internal oscillation, evidenced by temporal variations in levels of core clock proteins and expression of core clock genes. Furthermore, 3T3-L1 adipocytes responded circadian time-differently when stimulated with LPS and insulin. Anti-inflammatory effect of pioglitazone at ZT19 was more effective than that at ZT7, while no significant insulin-sensitizing effect was seen at both time points.

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NOMENCLATURE

6PFK1	6-phosphofructo-1-kinase
AMPK	5’ Adenosine Monophosphate-activated Protein Kinase
BMAL	Brain and Muscle-ARNT-like
CLOCK	Circadian Locomotor Output Cycles Kaput
CRY	Cryptochrome
Ctrl	Control
DMEM	Dulbecco’s Modified Eagle’s Medium
ER	Endoplasmic Reticulum
F2,6BP	Fructose-2,6-bisphosphate
FBS	Fetal Bovine Serum
FFAs	Free Fatty Acids
G3P	Glycerol-3-phosphate
GLUT4	Glucose Transporter 4
HGP	Hepatic Glucose Production
IKK	IκB Kinase
IL-6	Interleukin-6
IL-10	Interleukin-10
iPFK2	Inducible 6-phosphofructo-2-kinase
IRS	Insulin Receptor Substrate
JNK	c-Jun N-terminal Kinase

KD	Knockdown
LPS	Lipopolysaccharide
MCP-1	Monocyte Chemoattractant Protein-1
NF-κB	Nuclear Factor κB
PBS	Phosphate-Buffered Saline
PER	Period
PFK-1	Phosphofructokinase-1
PFK-2/FBPase	Phosphofructokinase-2/fructose-2,6-bisphosphatase
PI3K	Phosphatidylinositol-3 Kinase
Pio	Pioglitazone
PPAR	Peroxisome Proliferator-activated Receptor
Rev-erbα	Reverse Erythroblastosis Virus α
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gels
SCN	Suprachiasmatic Nucleus
T2DM	Type 2 Diabetes Mellitus
TG	Triglyceride
TLR4	Toll-like Receptor 4
TNFα	Tumor Necrosis Factor α
TZD	Thiazolidinediones
WAT	White Adipose Tissue

TABLE OF CONTENTS

LIST OF FIGURES

Page

1. INTRODUCTION

Adipose tissue plays an important role as an endocrine organ in metabolic control through the synthesis and secretion of adipokines. Studies have shown that chronic inflammation of the adipose tissue is highly related to insulin resistance as well as metabolic disorders including obesity and Type 2 Diabetes Mellitus (T2DM) [1].

With the increasing incidence of T2DM in the last three decades, metformin, as one of the first-line clinical anti-diabetic oral drugs for T2DM, has been shown to have beneficial effects including decreasing hepatic glucose production, improving insulin sensitivity, and suppressing inflammation, primarily in the liver though AMP-activated protein kinase (AMPK) pathway [2, 3]. However, the effect and mechanism of metformin on adipose tissue and adipocytes has not been fully investigated, as well as the role of PFKFB3/iPFK2, the gene that has been shown to be protective against insulin resistance and adipose inflammatory response caused by overnutrition [4], in the beneficial effects of metformin.

At the same time, a growing body of evidence has suggested that a disruption in circadian biological clock can contribute to the increased prevalence of obesity and T2DM [5, 6]. Adipose tissue, as one of the major peripheral tissues that display oscillations of circadian rhythms [7, 8], plays an essential role in metabolic regulation and exhibits insulin resistance and inflammatory response in overnutrition-related diseases such as obesity and T2DM [9, 10].

Therefore, the major goal of this study is to investigate the involvement of PFKFB3/iPFK2 in metformin actions using 3T3-L1 adipocytes, on the aspect of anti-inflammatory effect and improvement in insulin sensitivity; and to identify the circadian rhythm in adipocytes and whether treating adipocytes with drugs at different circadian times could result in differential beneficial effects.

2. LITERATURE REVIEW

2.1 Inflammation, insulin resistance, and T2DM

Inflammation has been categorized into two subtypes based on its characteristics. When injured, the body responses to the injury through acute inflammation with symptoms including swelling, redness, fever and pain. This is the classic type of inflammation that is a short-term adaptive action that is essential for tissue repair and tissue regeneration [11]. In recent years, the other type of inflammation, low-grade chronic inflammation has been studied more heavily for that it is metabolically triggered inflammation that ties closely with metabolic disorders such as obesity, T2DM and cardiovascular diseases [11, 12].

Low-grade chronic inflammation is displayed with increased inflammatory cytokines production, abnormal secretion of adipokines, as well as elevated plasma lipid levels [12, 13]. The association between chronic inflammation and obesity was first evidenced about three decades ago by the overexpression of proinflammatory cytokine tumor necrosis factor-α (TNF-α) in adipose tissue of obese mice [14], and in the adipose tissue and muscle in obese humans [12, 15]. In later research, this close association between obesity and inflammatory response has been further indicated in obese animal models and human studies, evidenced by activation of inflammatory signaling pathways with elevated plasma levels of inflammatory cytokines such as TNF-α and interleukin (IL)-6, increased production and secretion of leptin from adipose tissue, as well as high

levels of other inflammatory markers [16-18]. The expression levels of these major factors link metabolic regulation together with inflammatory responses.

An overproduction of inflammatory cytokines contributes to insulin resistance. Insulin is a crucial regulatory hormone which facilitates glycolysis and uptake of glucose by peripheral tissues, and stimulates lipid synthesis and decreases triglyceride (TG) breakdown [19]. Insulin resistance is a condition when these peripheral tissues, including liver, adipose tissue and skeletal muscle, cannot respond to insulin normally for it to perform its regulatory action in nutrient metabolism, which is a key contributor to metabolic disorders [1, 19].

In healthy individuals, insulin functions upon binding to insulin receptor on the cell membrane in target tissues. Insulin receptor will become phosphorylated and also stimulates the tyrosine phosphorylation of some other substrates including insulin receptor substrate (IRS) proteins, which will then activate Phosphatidylinositol-3 Kinase (PI3K)-Akt signaling pathway, a crucial step in mediating downstream insulin signaling action [11, 12, 20]. In adipocytes and muscle cells, phosphorylation of Akt stimulates intracellular insulin-responsive glucose transporter 4 (GLUT4) to translocate to cell surface to enhance the uptake of glucose [21-23]. Inhibitory serine phosphorylation of IRS-1 negatively impacts the tyrosine phosphorylation and the reaction to insulin of IRS-1.

Obesity-associated high levels of TNF-α, free fatty acid (FFA) and endoplasmic reticulum (ER) stress will activate inflammatory kinases including c-Jun N-terminal kinase (JNK) and IκB kinase (IKK). JNK, and possibly IKK, will stimulate the

inhibitory serine phosphorylation of IRS-1 to inhibit insulin signaling pathway [11, 12, 19]. JNK activation further stimulates increased cytokine levels such as IL-1β and TNF-α. Other than possible phosphorylation of IRS-1 on serine residues and impairment of islet cell function, IKKβ also activates Nuclear Factor κB (NF-κB), which is an important transcription factors that triggers production of several inflammatory mediators through synthesis of its target gene products [24]. At the same time, ER stress generated by overnutrition will cause high production level of reactive oxygen species (ROS) and leads to oxidative stress, the feature underlying obesity, to enhance inflammatory response and exacerbate insulin resistance [11, 12]. This mechanism of the causative link between inflammatory pathways and insulin resistance is illustrated in Figure 1.

2.2 Adipose tissue and inflammatory response

In the traditional notion, adipose tissue simply serves as energy storage pool which stores and releases energy when needed. However, in addition to its inert function of energy storage, adipose tissue is now commonly considered a dynamic endocrine organ that plays a regulatory role in metabolic homeostasis. It functions through the production and secretion of adipokines including leptin, adiponectin and resistin, as well as proinflammatory and anti-inflammatory cytokines and chemokines including TNF-α, IL-6, IL-10 and CPT1 [13].

A lot of evidence has suggested that adipose tissue plays an important part in metabolic disorders and that adipose dysfunction ties obesity closely to the pathogenesis

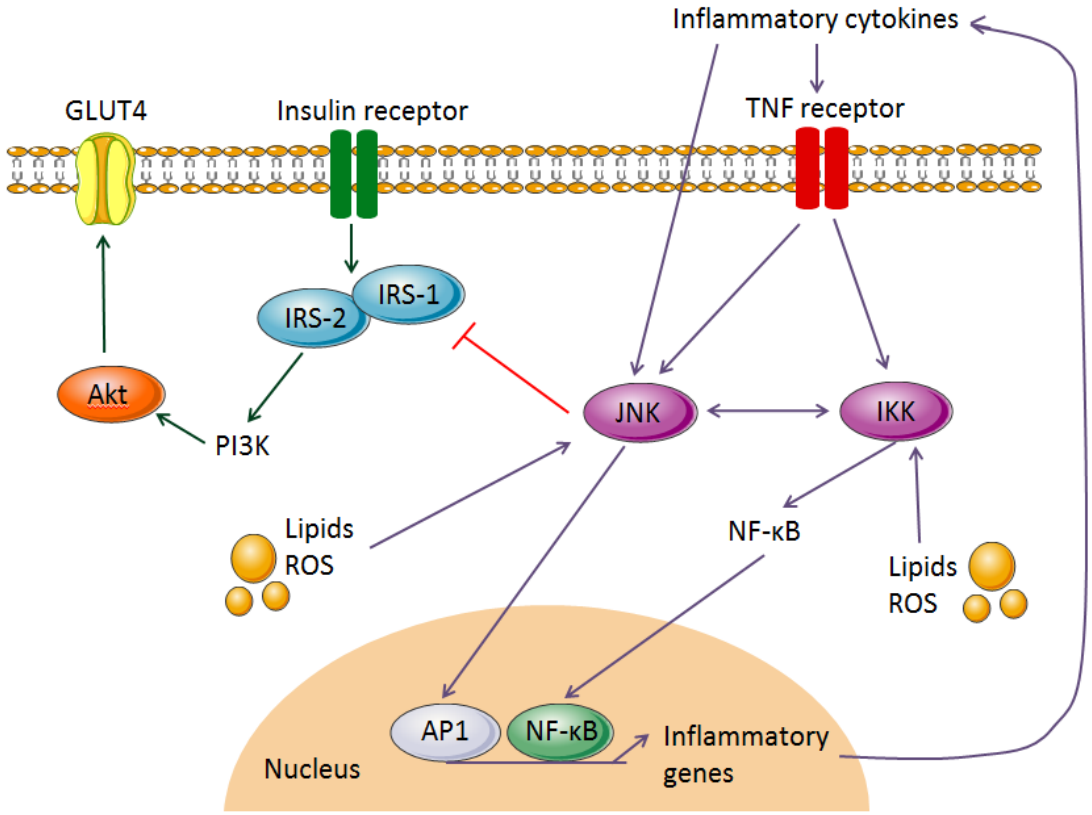

Figure 1. Molecular pathway integrating inflammatory response with insulin action. [11, 12, 20]

of T2DM through development of insulin resistance. When obesity occurs due to nutrient surplus, the inflammatory response predominantly resides in the adipose tissue, where inflammatory mediators are generated [11]. Therefore, adipose tissue has been studied extensively in the aspect of metabolic regulation.

As an individual becomes obese, the adipocytes will enlarge and circulating FFA and glycerol levels are elevated due to increased breakdown of TG [25]. Increased FFAs

will reduce glucose uptake and glucose oxidation, increase hepatic glucose output, as well as inhibiting insulin signaling through serine phosphorylation of IRS-1 [23]. Leptin is a peptide hormone secreted by adipocytes, and its primary role is appetite control. Another hormone secreted by adipose tissue is adiponectin, which can enhance insulin sensitivity and reduce inflammatory response through decreasing TNF-α production and activity, inhibiting IL-6 production, and stimulating FFA oxidation and anti-inflammatory IL-10 expression. Even though it is secreted predominantly by adipocytes, adiponectin levels tend to decrease with increased fat mass in obese individuals; in patients with T2DM, adiponectin levels decreases significantly [9, 13]. Resistin is another adipocyte-secreted molecule which has recently been found to be increased with obesity and indicated to be proinflammatory through multiple possible mechanisms [26].

As mentioned above, TNF-α is overexpressed in white adipose tissue (WAT) in obese subjects, and can activate inflammatory cascade and act as inhibitors of the insulin signaling pathway directly via inhibitory phosphorylation of IRS-1 and indirectly via activation of JNK and NF-κB inflammatory pathway. There is some evidence that TNF-α may have impact on downregulation of adiponectin production. In addition to TNF-α, proinflammatory markers that WAT produces with the enlargement of adipocytes include cytokines like IL-1β and IL-6, and chemokines such as monocyte chemoattractant protein-1 (MCP-1) [27-29]. Diabetic patients have been shown to have elevated IL-6 levels, which is associated with impairment in insulin sensitivity. Even though IL-6-deficient mice exhibit insulin resistance and diabetes, IL-6 is still commonly considered as a proinflammatory cytokine. In insulin-resistant mice, IL-1β

mRNA expression in epididymal fat and circulating IL-1β levels are increased [30]. MCP-1 mRNA expression has been shown to increase in obese mice in response to adiposity, and contribute to insulin resistance and macrophage infiltration in adipose tissue [31]. Toll-like receptor-4 (TLR4) is an immune sensor that has been reported to increase in obese tissues to induce inflammatory response. FFAs are considered to contribute to TLR signaling [32]. Other than adipocytes, macrophages are also a component of adipose tissue and are accumulated in the presence of obesity. Like adipocytes, macrophages contribute to the upregulation of TNF-α and IL-6 as well [13]. However, this thesis will mainly focus on the adipocytes. The metabolism described above is illustrated in Figure 2.

The most widely utilized cell lines in the study of adipocytes are 3T3-L1 and 3T3-F442A, which are isolated from mice embryos. Mature adipocytes are differentiated from fibroblast-like preadipocytes through differentiation stage under inducing agents. At confluence, preadipocytes will gradually experience morphological changes and convert into round shape, accumulate lipid droplets, as well as display biological characteristics such as gene expression of mature adipocytes of live adipose tissue [33, 34].

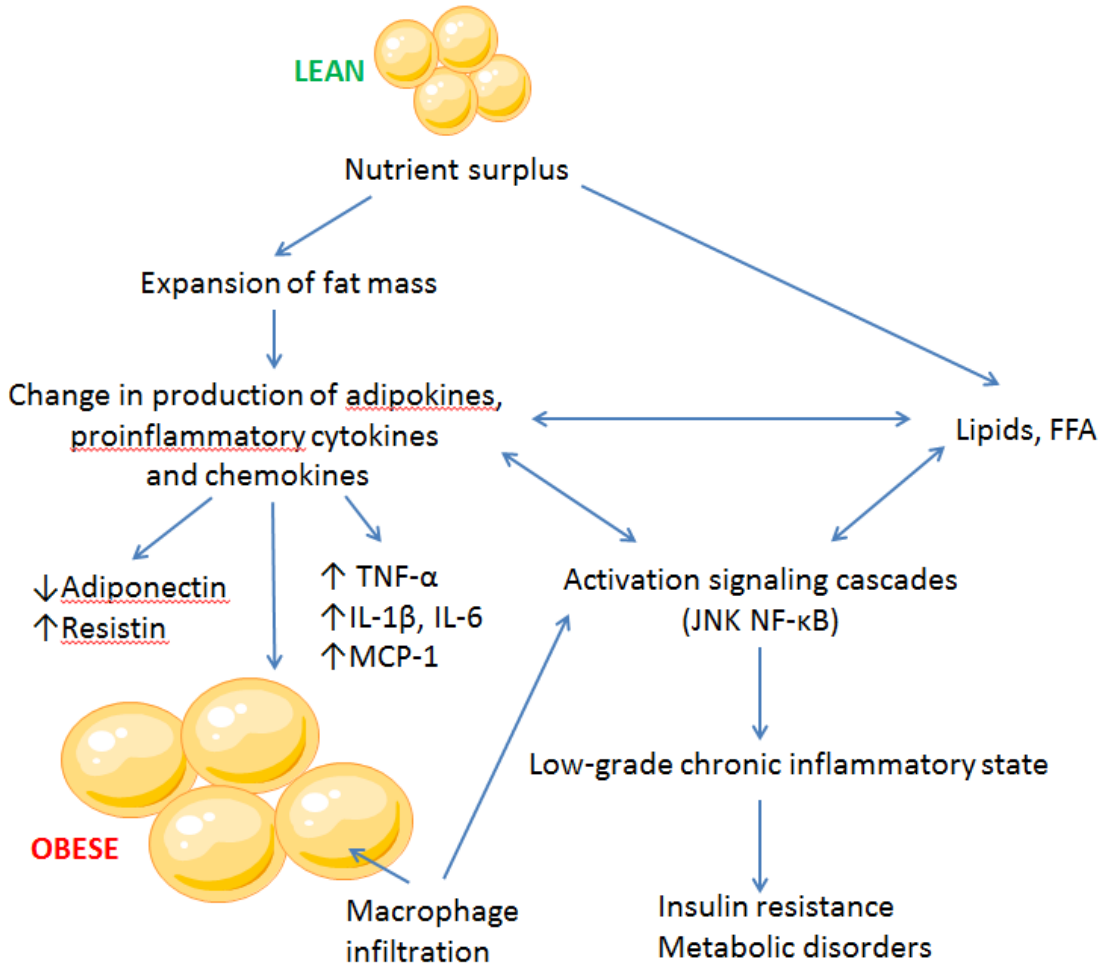

Figure 2. Potential mechanism of adipose tissue inflammation in overnutrition-induced obesity. [11, 29, 30]

2.3 Metformin, pioglitazone and T2DM

2.3.1 Metformin

Metformin has been one of the most widely used first-line clinical anti-diabetic oral drugs for T2DM starting from its initial medical use. In clinical trials, obese patients who are given metformin have attained lower fasting plasma glucose and lower levels of

HbA_{1c} [35]. Metformin is also shown to be effective in preventing or at least delaying progression of diabetes and associated complications among people who are at high risk [2, 36]. In addition to ameliorating insulin resistance, the beneficial effects of metformin in targeting inflammation could be utilized as a preventative and therapeutic approach to treatment in diabetes and its complications [37].

Currently, most studies on metformin focus on its action in the liver, with its effect in reduction of HGP and increase in peripheral glucose uptake with the activation of AMPK [3, 38]. However, the effect of metformin in one of the major insulin-sensitive tissue, adipose tissue, has not been fully investigated and remains controversial. In perivascular adipose tissue, metformin served as an AMPK-activating agent, suppressed inflammatory response through inhibition of NF-κB phosphorylation and expression of proinflammatory adipocytokines [39]. In some studies, metformin was able to increase adiponectin expression level *in vivo* and *in vitro* in subcutaneous adipose tissue [40]. Yet in another study, metformin was shown to reduce adiponectin protein expression, and activated AMPK in 3T3-L1 adipocytes [41]. Meanwhile, when high-fat diet-fed mice were treated with metformin, there was no significant improvement in AMPK activation and inflammatory response in the adipose tissue [42].

2.3.2 Pioglitazone

Pioglitazone is among the thiazolidinediones (TZDs) class that are insulin-sensitizing agents clinically used to treat patients with T2DM. Similar to metformin, pioglitazone does not stimulate pancreatic islet cells to secrete more insulin [38]. TZDs act through their binding to a specific nuclear transcription factor peroxisome

proliferator activated receptor (PPAR)-γ, which is highly expressed in the adipose tissue, and in turn take action in insulin sensitivity, lipid metabolism and inflammatory response. In adipose tissue, TZDs have been shown to stimulate lipogenesis, glucose uptake, fatty acid uptake and glucose oxidation, and suppress insulin-stimulated lipolysis [43]. In addition, activation of PPAR-γ accounts for suppressed NF-κB to inhibit production of proinflammatory cytokines [11]. It has been established that pioglitazone treatment can ameliorate insulin resistance and improve glycemic control and lipid profile, despite an increase in body fat mass [44].

2.4 PFKFB3/iPFK2 and adipocyte physiology

PFKFB3 is a gene that encodes inducible 6-phosphofructo-2-kinase (iPFK2), which is an inducible isoform of 6-phosphofructo-2-kinase/fructose-2,6-bisphotase (PFK-2/FBPase). PFK-2/FBPase regulates the production and degradation of fructose 2,6-bisphosphotate (F2,6BP), which is a potent allosteric activator of 6-phosphofructo-1-kinase (6PFK1), the rate-limiting enzyme for glycolysis pathway [45-48]. Therefore, PFKFB3/iPFK2 takes an important part in regulating and stimulating glucose metabolism.

Studies have shown that PFKFB3/iPFK2 is highly expressed in adipose tissue of human and mice and plays a role in metabolic regulation. With glucose uptaken into adipocytes, PFKFB3/iPFK2 stimulates the synthesis of F2,6BP, which in turn activates 6PFK1 potently. This catalyzes the production of glycolytic products: glycerol-3-phosphate (G3P) and pyruvate. Pyruvate metabolism generates acetyl-CoA, which will

be used for FFA synthesis. FFAs digested from diet transport into the cells through fatty acid transporters. Together with FFA generated from acetyl-CoA and G3P, they are converted to triglycerides to be stored in the adipocytes/adipose tissue [49]. This regulatory function of PFKFB3/iPFK2 is illustrated in Figure 3.

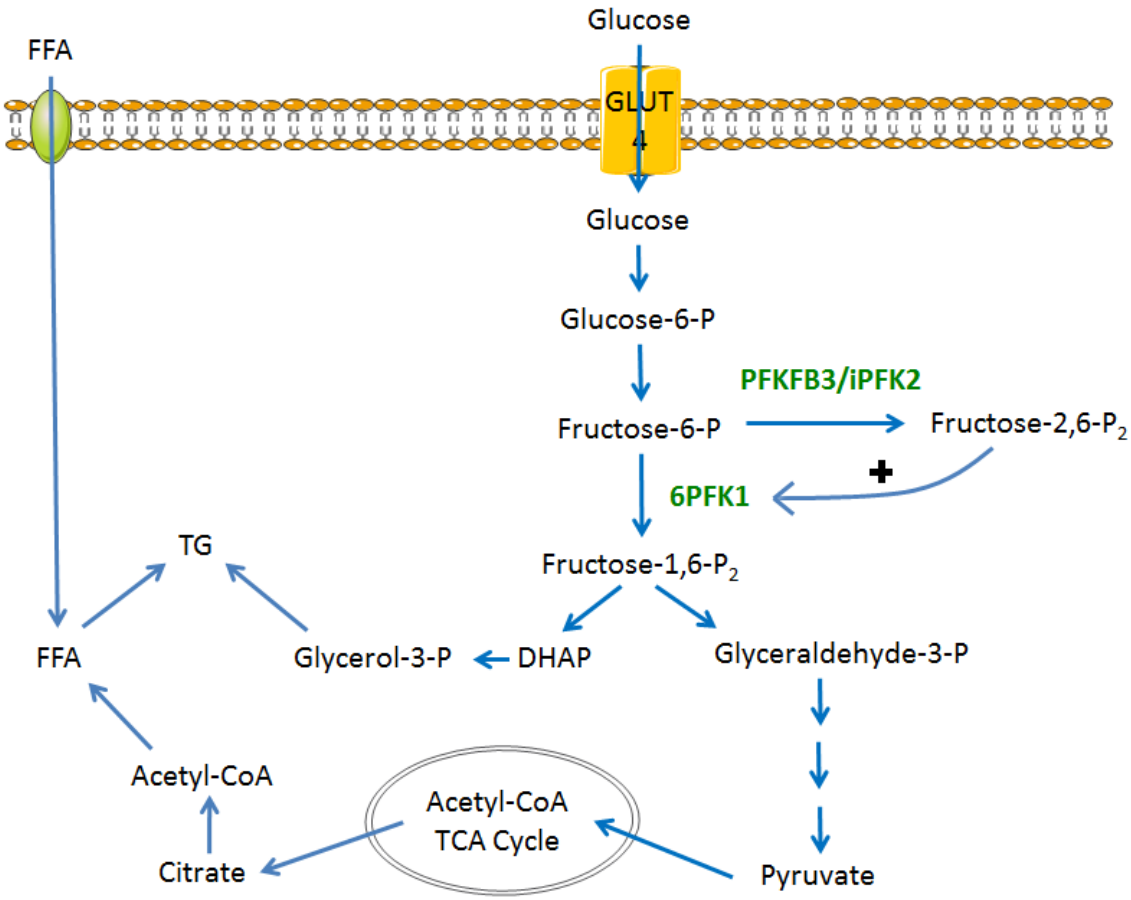

Figure 3. PFKFB3/iPFK2 regulates adipocyte glucose and lipid metabolism.
Modified based on Guo, et al. [49]

In support of this mechanism, studies suggested that under high-fat diet, a disruption of PFKFB3/iPFK2 in mice led to less weight gain in comparison to wild-type littermates, yet resulted in more severe adipose tissue dysfunction, adipose inflammatory response, and systemic insulin resistance. A knockdown of PFKFB3/iPFK2 in 3T3-L1 adipocytes showed similar outcomes evidenced by activated JNK and NF-κB pathways, increased proinflammatory cytokine expression including TNF-α and IL-6, decreased adiponectin expression and decreased insulin signaling, suggesting that PFKFB3/iPFK2 serves as a link between adipocyte nutrient metabolism and inflammatory response [4].

When PFKFB3/iPFK2 is disrupted, glycolysis is suppressed due to lack of production of the rate-limiting enzyme 6PFK1. As a result, lipogenesis and synthesis and storage of triglyceride using glucose as a source are impaired. Meanwhile, this leads to a compensatory elevated fatty acid oxidation, contributing to increased ROS production, which is associated with elevated proinflammatory cytokine expression through phosphorylation of JNK1 and NF-κB [4]. Therefore, PFKFB3/iPFK2 contributes to the anti-inflammatory response in adipocytes through regulation of fuel metabolism and inhibition of undesired high levels of fatty acid oxidation.

2.5 Circadian rhythm in adipocytes

The metabolism of adipose tissue is not simply affected by dietary factors alone, but also environmental and intrinsic factors, and circadian rhythm is one of them. In order to adapt to the 24-hour light and dark cycle in the external environment to optimize

activities, mammals have developed self-sustaining endogenous biological clocks named internal circadian clocks.

A great amount of evidence has discovered an association between circadian rhythm and metabolism, and that circadian biology is an underlying factor in the epidemic occurrence of obesity and diabetes, other than excessive dietary intake and lack of physical exercise habits of the population. Night-shift workers have been found to display higher BMI, exaggerated postprandial glucose response and abnormal blood lipid profile, and are more likely to develop metabolic disorders [50-52]. People with a typical sleep duration of less than 6 hours more than 9 hours experience higher rate of impaired glucose tolerance and higher prevalence of T2DM [53]. Interestingly, proinflammatory cytokine levels of TNF-α and IL-6 in the morning are significantly higher in patients with apnea [54]. *Clock* mutant mice are obese, exhibit disrupted feeding rhythm, and develop hyperlipidemia, hyperglycemia, and other metabolic syndrome [55]. In rodents and humans, an attenuation of circadian control of glucose metabolism and insulin action may promote T2DM and incidence of other metabolic disorders; while in some tissue of diabetic animals, oscillation of circadian clock genes are altered [5, 6].

At the molecular level, circadian clocks are governed by a self-sustaining transcriptional and translational feedback loop involving core circadian clock genes, which activate or suppress their own expression. These core clock genes include circadian locomotor output cycles kaput (*Clock*), brain and muscle-ARNT-like 1 (*Bmal1*), Period1 (*Per1*), Period2 (*Per2*), Period3 (*Per3*), Cryptochrome1 (*Cry1*), and

Cryptochrome2 (*Cry2*). *Clock* and *Bmal1* encode for CLOCK and BMAL1 proteins, which will form a heterodimer complex that serves as the positive limb of the feedback loop and drives the oscillatory function of the circadian clock. Upon binding to the enhancer E-box sequence in the promoter region, the CLOCK:BMAL1 heterodimer will activate transcription of repressor genes including *Per1*, *Per2*, *Per3*, *Cry1*, and *Cry2*, which contribute to the negative limb of the feedback loop. When high levels of PERs and CRYs are produced and released into the cytoplasm, they dimerize and transfer to the nucleus to suppress CLOCK-BMAL1-stimulated transcription. Because of the continuous operation of the regulatory feedback loop, the circadian mechanism oscillates with a rhythm. This core feedback loop is supported by another ancillary loop that is regulated by reverse erythroblastosis virus (*Rev-erbα*), which suppresses *Bmal1* expression (Figure 4) [6, 56-60].

Besides central circadian clock which exists in the suprachiasmatic nucleus (SCN) in the hypothalamus, peripheral clocks are located in peripheral tissues such as liver, adipose tissue, pancreas, heart and skeletal muscle. By interacting with each other, central and peripheral clocks regulate eating behavior and metabolic functions in these key organ systems [61]. The existence of a fully functional circadian clock within the adipose tissue has been investigated in several studies, indicated by the oscillation of the expression of core clock genes, including *Bmal1*, *Clock*, *Per1*, *Per2*, *Cry1*, *Cry2*, and *Rev-erbα* [7, 62, 63]. Disruption of *Bmal1* reduced adipocyte differentiation and lipid storage in adipocytes *in vivo* and *in vitro*, and mice lack of Per2 displayed disrupted lipid metabolism and lipid profile. More studies have presented that in the adipose tissue in

humans and mice models, circadian clock genes play a role in the regulation of adiposity, adipogenesis, lipogenesis, fatty acid oxidation, as well as lipolysis [61-65]. Therefore, it is meaningful to discover the circadian rhythms in adipocytes and investigate whether treating adipocytes at different circadian times could generate differential effects in order to get implications on time of drug delivery in regarding to obesity and diabetes.

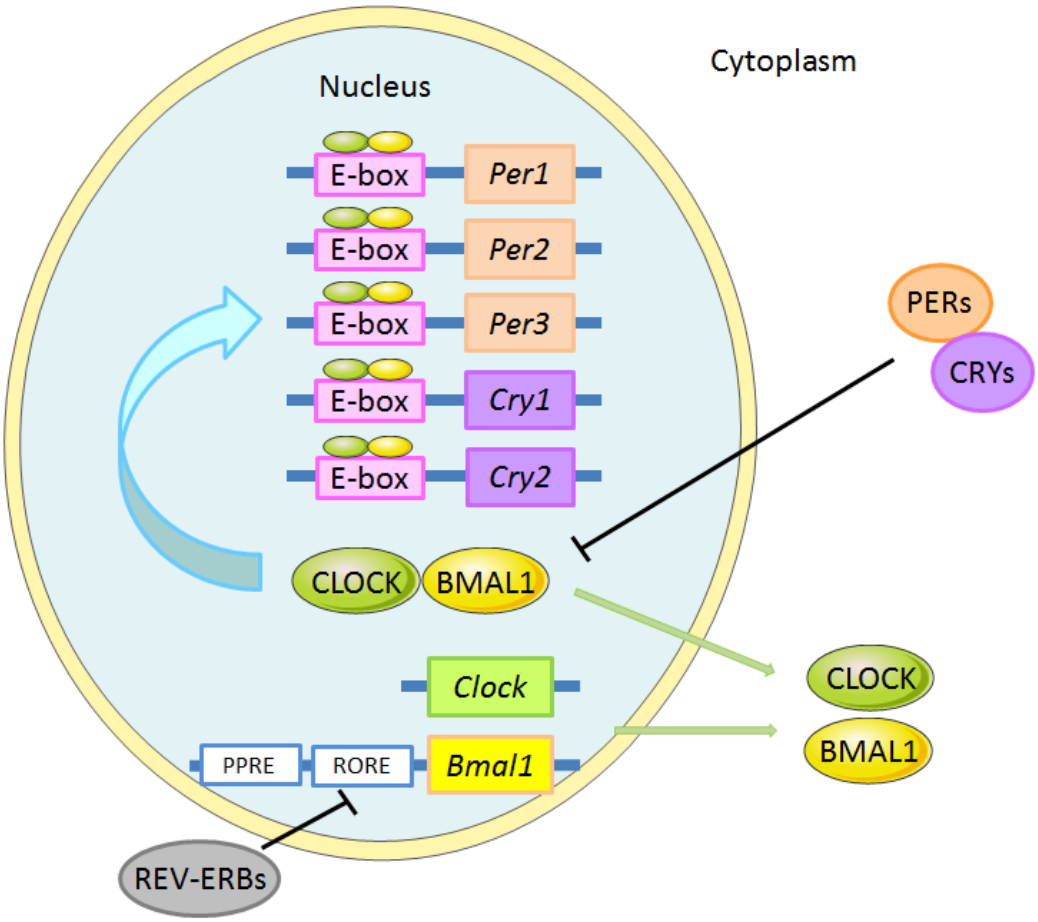

Figure 4. Transcriptional-translational feedback loop of core clock genes. [56, 60]

3. MATERIALS AND METHODS

The hypothesis of this study is that PFKFB3/iPFK2 is involved in the beneficial effects of metformin treatment in adipocytes on the aspects of insulin resistance and inflammatory response, and treating adipocytes with pioglitazone at different circadian times lead to different extent of effects on insulin resistance and inflammatory response.

The objectives of this study are: 1. Determine the beneficial effect of metformin on adipocytes; 2. Determine the involvement of PFKFB3/iPFK2 in effect of metformin and its possible mechanism; 3. Determine the circadian rhythm in adipocytes; 4. Determine the different effect of pioglitazone at different circadian times.

3.1 Cell culture

Wild-type 3T3-L1 adipocyte cell line was used in this study, as well as stable PFKFB3/iPFK2-knockdown (iPFK2-KD) and PFKFB3/iPFK2-Control (iPFK2-Ctrl) 3T3-L1 adipocytes. Preadipocytes were first cultured in high glucose Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C with 5% CO_2 in a humidified incubator. Fresh medium was changed every other day until cells were confluent.

Two days after the cells became confluent, the differentiation was induced with growth medium containing 10 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (designated Day 0) for 48 hours. Then, cells were maintained in growth medium with 1 μg/ml insulin, with medium being refreshed every other day

for additional 4 days, followed by another incubation of 48 hours in growth medium without supplementation of insulin. Cells were treated on Day 8 after full differentiation.

3T3-L1 adipocyte cell lines were previously established in Dr. Chaodong Wu's lab. Stable iPFK2-KD and iPFK2-Ctrl cell lines were generated by the transfection of predifferentiated 3T3-L1 cells with plasmids containing shRNA against mouse PFKFB3 and shRNA vector respectively with Lipofectamine 2000 Transfection Reagent following manufacturer's protocol [4]. To confirm the knockdown of PFKFB3/iPFK2, western blot was used to examine PFKFB3/iPFK2 expression for both iPFK2-KD and iPFK2-Ctrl adipocytes.

3.2 Cell treatment

3.2.1 Metformin experiments

Different treatments were performed for determination of the best dose and time course for metformin on adipocytes. 5 μM, 50 μM and 500 μM of metformin were selected to be added to differentiated 3T3-L1 adipocytes for the best dose under stimulation of lipopolysaccharide (LPS). After determining the best dose, metformin was treated for different lengths of time (0 hr, 1 hr, 6 hr, 24 hr and 48 hr) for the most effective time course under LPS stimulation. Metformin and LPS were prepared with PBS as solvent, so PBS was used as control group, and was considered as 0 μM and 0 hr of metformin treatment. LPS was added 30 min before harvest for protein samples at 100 ng/ml, and 6 hours before harvest for RNA samples at 20 ng/ml. All cells were harvested at the same time.

After the confirmation of the most effective dose and time course of metformin treatment, metformin was used to treat fully differentiated iPFK2-KD and iPFK2-Ctrl adipocytes. Cells were divided into different sets to investigate inflammatory response and insulin signaling response. For inflammatory study, 50 μM metformin was added for 24 hr to iPFK2-KD and iPFK2-Ctrl cells, and same volume of PBS was added in another set as control. 100 ng/ml LPS was added to signal inflammatory response, and same volume of PBS was added as control. LPS was added 30 min before harvest for protein samples, and 6 hours before harvest for RNA samples. For insulin signaling response, 50μM metformin and PBS were added for 24 hr to iPFK2-KD and iPFK2-Ctrl cells, and 100 nM insulin and PBS were added 30 minutes before harvest for protein samples.

After each treatment, cells were washed twice with cold PBS before harvest for protein samples. Protein samples were harvested with cell lysis buffer (20 mM Hepes, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 1% NP-40, 0.1% SDS, 2 mM EDTA, 2 mM Na Vanadate, pH 7.4), and RNA samples were harvested with RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) following manufacture's protocol, without washing with cold PBS. Protein samples were analyzed with western blot, and RNA samples were analyzed with RT-PCR as described in the following.

3.2.2 Circadian experiments

After reaching full differentiation, adipocytes needed to be synchronized by a change of growth medium to DMEM with 50% horse serum for a serum shock. After 2 hr, this medium was changed to DMEM containing 5% FBS for 1 hr, followed by a change to DMEM supplemented with 10% FBS for 2 hr. After 2 hr, this was designated

to be ZT 0, where the medium was changed back to growth medium. Protein and RNA samples of adipocytes were harvested at ZT 1, ZT 7, ZT 13, and ZT 19 for an analysis of the existence of circadian rhythm within adipocytes.

After examining the circadian rhythm within adipocytes, 100 ng/ml pioglitazone (Pio) was treated to synchronized cells at ZT 7 and ZT 19 for 48 hr, and same volume of DMSO was added as control because Pio was dissolved in DMSO. To investigate inflammatory response and insulin signaling response, 100 ng/ml LPS and 100 nM insulin were added 30 min before harvest, with PBS being control. Protein samples were harvested with cell lysis buffer, and were then analyzed with western blot as described below.

3.3 Protein isolation and preparation of protein samples

After protein samples were harvested with cell lysis buffer, they were left frozen overnight at -80°C. Protein samples were purified by getting the supernatant after centrifuge at 4°C at 14,000 rpm. The concentration of the samples was measured with Micro BCA Protein Assay Kit (Thermo scientific, USA) and was quantified with infinite M200 (Tecan, Switzerland) at 562 nm absorbance. Samples with the same protein concentration were prepared with addition of lysis buffer, loading buffer and DTT, and were boiled for 5 min before storage at -80°C.

3.4 Western blot analysis

Protein samples were loaded to 8% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) to be separated by electrophoresis, and then the gels were transferred onto PVDF membranes at 25V for 30 min. The membranes were blocked with 5% skim milk in TBS for 4 hrs, and incubated with a certain primary antibody at 4°C overnight. Primary antibodies were diluted 1000 times with 5% BSA in TBS. After 3 times of washing with TBS, membranes were incubated in a 1:7,500 goat anti-rabbit secondary antibody for 1.5 hrs. After another 3 times of 10 min wash, membranes were detected with Western chemiluminescent reagent under CCD Camera (Cascade II:512, Photometrics, Tucson, AZ). The levels of JNK, phospho-JNK (p-JNK), NF-κB (p65), phospho-NF-κB (Pp65), Akt, phosphor-Akt (p-Akt), AMPK, phospho-AMPK (p-AMPK) and PFKFB3 were analyzed for both studies, with circadian studies measuring additional CLOCK and Bmal1. GAPDH was used as a total control.

3.5 RNA isolation and RT-PCR

Total RNA of cells were extracted from harvested cells using STAT-60. RNA concentrations were measured using Nanodrop 2000 Spectrophotometer (Thermo Scientific) and reverse transcribed into cDNA samples accordingly with GoScriptTM Reverse Transcription System (Promega). The relative expression levels were measured through RT-PCR using Power SYBR Green under Roche LightCycler II. The mRNA levels analyzed included PFKFB3, TNF-α, IL-1β, IL-6, adiponectin, TLR-4, resistin, and MCP-1 for metformin studies, and PFKFB3, CLOCK, BMAL1, Per1 and Per2 for

circadian studies. The variability in the mRNA expression level was normalized with ribosomal 18S RNA amplifications [66].

3.6 Statistical analysis

Statistical data were presented as means ± standard error (SE). Comparisons between two groups of data were analyzed by two-tailed Student's t-tests, where $p<0.05$ was considered significant. Significant differences at $p<0.05$, $p<0.01$ and $p<0.001$ compared to negative controls are indicated by "*", "**" and "***", or "†", "††", and "†††", respectively.

4. RESULTS AND DISCUSSIONS

4.1 Involvement of PFKFB3/iPFK2 in beneficial effect of metformin on adipocytes

4.1.1 Dose and time effect of metformin on adipocyte inflammatory response

To identify the most effective dose and time course of metformin treatment *in vitro*, wild-type 3T3-L1 adipocytes were treated with metformin at increasing concentrations at 5 μM, 50 μM and 500 μM, and for 0h, 1h, 6h, 24h and 48h. All the cells were harvested at the same time, and 100 ng/ml LPS was used to induce inflammatory response.

In absence of metformin, LPS induced inflammatory response through the phosphorylation of JNK1 (p46), JNK2 (p54) and NF-κB. When different doses of metformin were added to the cells, western blot analysis did not show much difference on different doses (Figure 5A), but different time courses showed significant difference. Interestingly, metformin treatment showed a rhythm in anti-inflammatory effect, with 1h, 24h and 48h being effective in reducing phosphorylation of JNK, and 6h being not effective. Under LPS induction, there was inhibition of phosphorylation of JNK1 and JNK2 with metformin treatment for 1h, 24h and 48h. 24h of metformin treatment seemed to suppress phosphorylation of NF-κB from the western blot results (Figure 5B). The quantification of the densitometry of their expressions was performed under ImageJ software. Metformin treatment groups were compared with LPS-Met 0h group to determine the anti-inflammatory effect of metformin. Adipocytes significantly decreased

p-JNK expression when treated with metformin for 1h, 24h and 48h (Figure 5C), and significantly decreased p-NF-κB expression with metformin for 24h (Figure 5D).

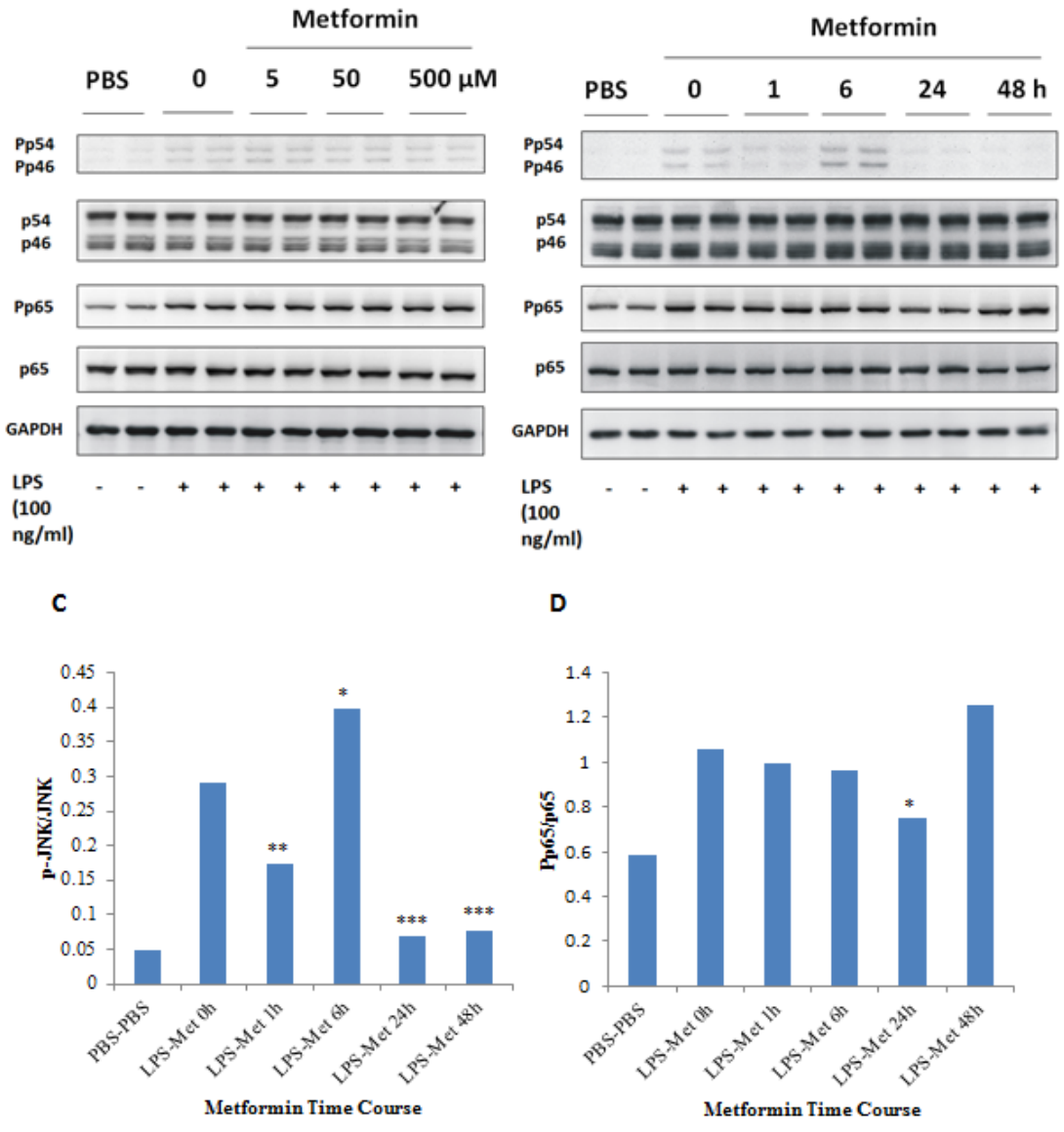

Figure 5. Metformin inhibits JNK phosphorylation time-dependently in 3T3-L1 adipocytes.

4.1.2 Dose-dependent and time-dependent effect of metformin on adipocytes gene and cytokine expression

In addition to the protein samples, RNA samples for dose and time treatments were harvested and analyzed with RT-PCR. Based on previous results, LPS-Met 1h group was not included in RT-PCR because of less significant differences. The mRNA expressions measured included proinflammatory cytokines TNF-α, IL-1β and IL-6, major adipokine adiponectin, and our interested gene PFKFB3. Their relative mRNA expression was determined with 18s as the control. Data are presented as mean ± SE (n = 3 unless specified otherwise). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for significance level in comparison between metformin dose treatment groups and LPS-Met 0 μM or LPS-Met 0h group.

As expected, the relative TNF-α expression increased when induced with LPS (LPS-Met 0 μM) compared to basal (PBS-PBS) in absence of metformin. This increase in TNF-α expression appeared to be ameliorated with treatment of metformin when at higher doses, with Met at 50 μM being significantly effective in reducing expression level of TNF-α (Figure 6A). Expression levels of IL-1β displayed similar pattern. The increase in relative IL-1β expression in LPS-Met 0 μM group was inhibited with higher doses of metformin treatment, with a concentration of 50 μM (n=2) being significantly effective. Meanwhile, low concentration of metformin of 5 μM led to a 2-fold increase in IL-1β expression compared to LPS-Met 0h group (Figure 6B). The relative expression levels of IL-6, however, did not exhibit similar results. IL-6 expression increased with induction of LPS as expected, but when metformin was added to adipocytes under LPS

stimulation, it not only showed limited effect in inhibiting IL-6 expression, but increased IL-6 expression significantly with increase in metformin concentration when compared with LPS-Met 0 μM group. This effect was strongest when the concentration of metformin was 500 μM (Figure 6C).

In adiponectin expression, it was expected that LPS induction will decrease adiponectin expression compared to basal, and metformin was expected to increase adiponectin relative expression compared to LPS-Met 0 μM group. However, metformin seemed to have limited effect on adiponectin expression. Due to the high standard errors within each group, the results of differences in adiponectin expression were not significant among different groups (Figure 6D).

Metformin treatment significantly increased PFKFB3 expression when at higher doses, with p-values of 0.00014 and 0.00015 for 50 μM and 500 μM metformin. PFKFB3 was highest expressed at 50 μM metformin (Figure 6E). This indicates that PFKFB3 expression is induced with effective metformin treatment, and that PFKFB3/iPFK2 may be involved in the action of metformin in adipocytes.

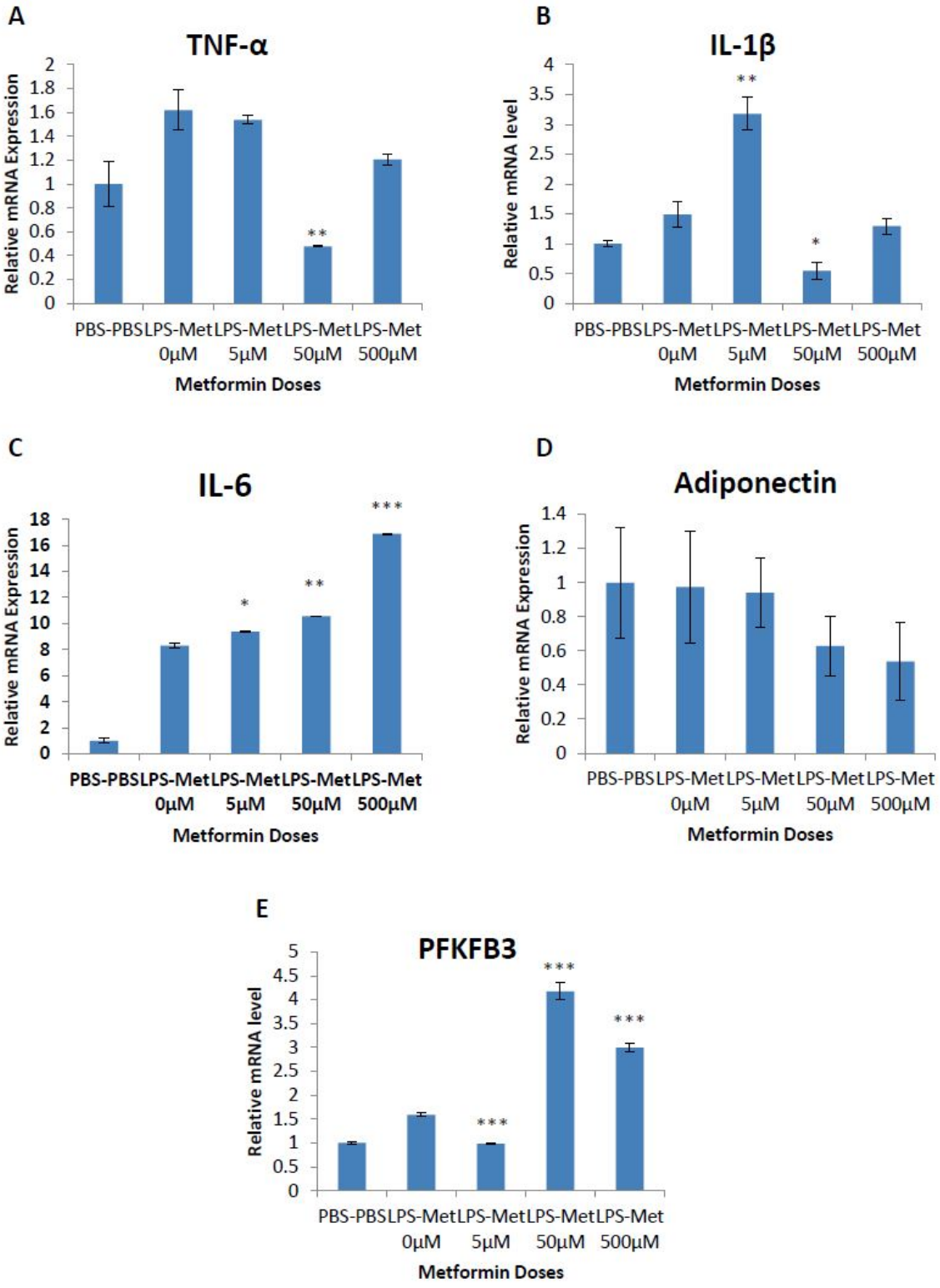

Figure 6. Dose-dependent effect of metformin on adipocyte gene expression.

The relative mRNA expression levels of proinflammatory cytokines for different metformin time courses were also examined through RT-PCR. Similar to the results in dose response, TNF-α and IL-1β displayed similar trend in regards to time response to metformin. In absence of metformin treatment, LPS induction stimulated upregulation in expression of proinflammatory cytokines TNF-α and IL-1β as expected. When metformin was added with the presence of LPS for all of these, this increase in cytokine expression was significantly inhibited. For both TNF-α and IL-1β, the inhibition of their expression was most effective when metformin was treated for 24 hr (Figure 7A and B). The expression level of IL-6 under metformin treatment was still to our surprise. Even though IL-6 expression increased under LPS induction when no metformin was treated, improvement was only seen when length of metformin treatment was 6h. Metformin did not show improvement for LPS-Met 24h and LPS-Met 48h groups, but increased IL-6 expression. IL-6 expression was significantly higher than expected, especially for LPS-Met 24h group (Figure 7C).

In adiponectin expression, LPS induction appeared to decrease adiponectin expression in absence of metformin as expected, but this change was not significant. Longer length of metformin treatment at 24h and 48h showed a trend in recovering the expression levels of adiponectin to basal levels, but due to standard errors, this improvement was not statistically significant (Figure 7D).

In different time courses of metformin treatment, PFKFB3 expression significantly increased compared to LPS-Met 0h group. PFKFB3 was highest expressed when metformin was treated for 24h (Figure 7E). The effect of metformin treatment on

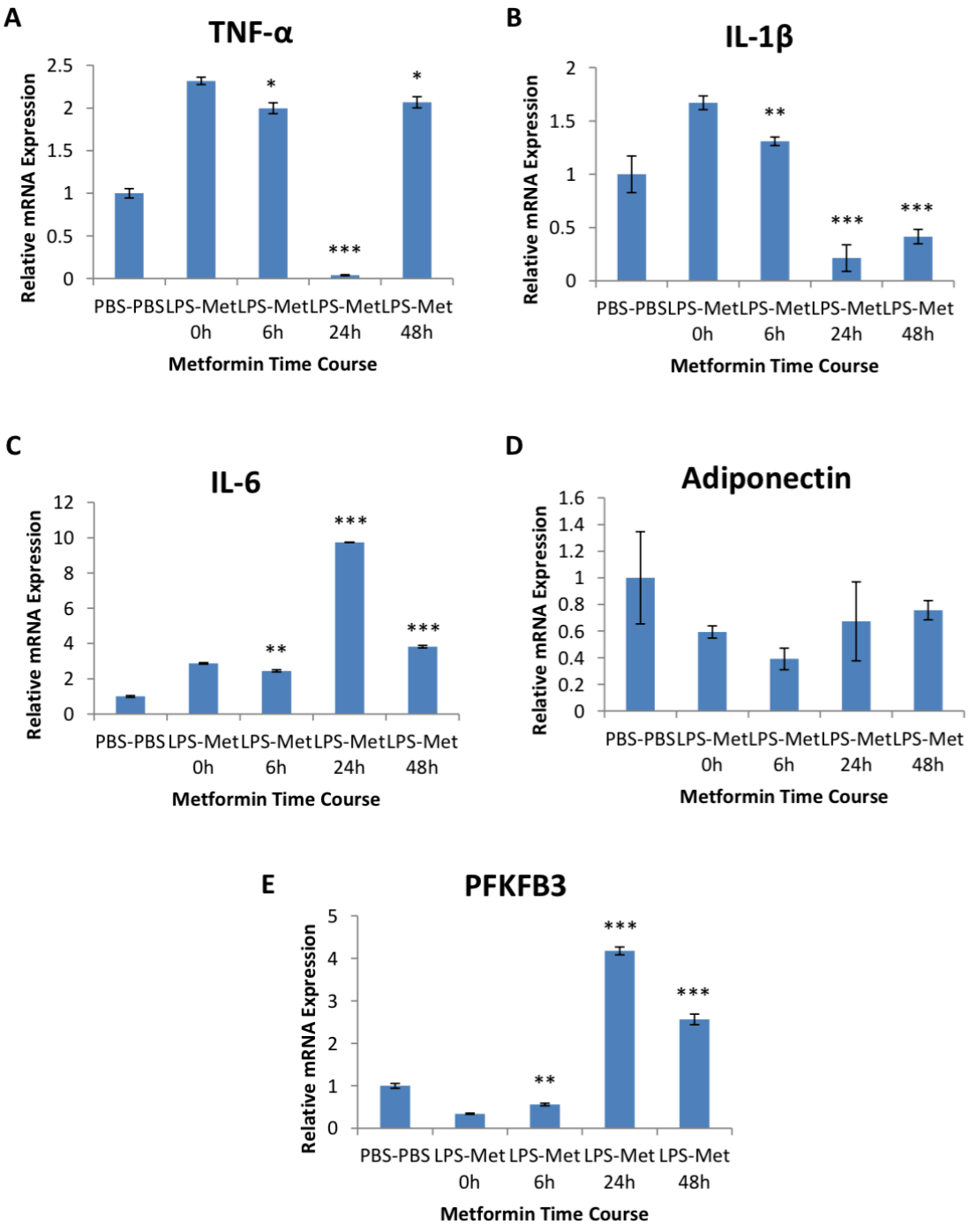

Figure 7. Time-dependent effect of metformin on adipocyte gene expression.

PFKFB3 expression coincided with dose and time course treatments, leading to our hypothesis that PFKFB3/iPFK2 is involved in effective metformin treatment.

When the results in the previous section and this section are taken together, when adipocytes are treated with metformin with a concentration of 50 μM for 24 hours, the results were most significant in the aspect of decreasing inflammatory response and proinflammatory cytokine expression. Therefore, in later experiment, metformin of 50 μM for 24 h was selected as the most effective dose and time to treatment of 3T3-L1 adipocytes.

4.1.3 Confirmation of the knockdown of PFKFB3/iPFK2 in adipocytes

After the determination of time and dose of metformin treatment, the involvement of PFKFB3/iPFK2 in the anti-inflammatory and insulin-sensitizing effect of the treatment in adipocytes was then explored with PFKFB3/iPFK2-Ctrl and PFKFB3/iPFK2-KD adipocytes. A western blot was first run to confirm the knockdown of PFKFB3/iPFK2. As expected, the knockdown of PFKFB3/iPFK2 was evidenced by significantly lower levels in iPFK2 in iPFK2-KD adipocytes compared to iPFK2-Ctrl adipocytes, regardless of the treatment. In iPFK2-Ctrl cells, PFKFB3/iPFK2 level was higher in PBS-Met group than that in PBS-PBS group after standardized with GAPDH, but this difference was not significant. Compared with that in iPFK2-Ctrl adipocytes, PFKFB3/iPFK2 did not increase, but tended to decrease with metformin treatment in iPFK2-KD adipocytes with a p-value of 0.0532 (Figure 8).

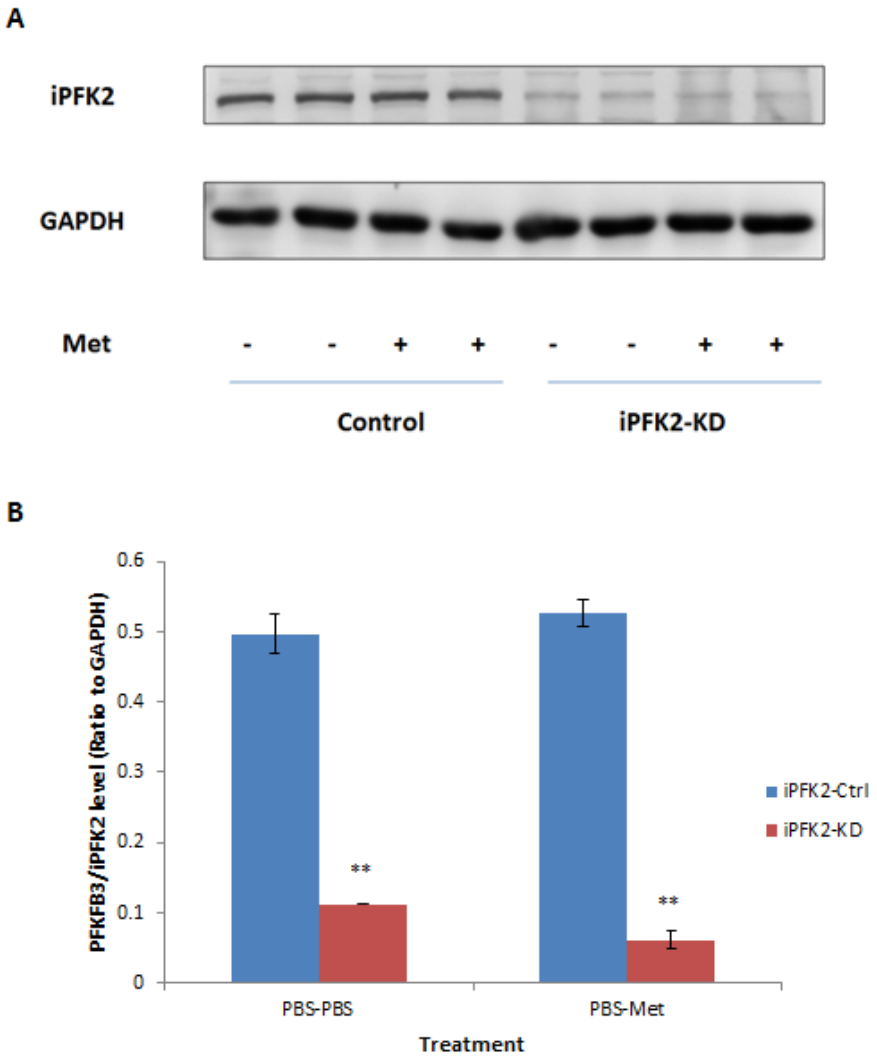

Figure 8. Confirmation of the knockdown of PFKFB3/iPFK2 in iPFK2-KD adipocytes.

4.1.4 A disruption of PFKFB3/iPFK2 in 3T3-L1 adipocytes could impair the anti-inflammatory effect of metformin

To study the role of PFKFB3/iPFK2 in metformin treatment, the difference in amelioration of inflammatory response under metformin treatment was first investigated. Both iPFK2-Ctrl and iPFK2-KD cells were treated with metformin for 24 hours with a concentration of 50 μM. Prior to harvest, inflammatory response was induced by LPS 30 minutes before harvest for protein samples, and 6 hours before harvest for RNA samples. In western blots for analysis of protein samples, changes in levels and phosphorylation states of JNK and NF-κB among different treatment groups were measured for iPFK2-Ctrl and iPFK2-KD adipocytes. mRNA levels of proinflammatory markers and adipokines were quantified with RT-PCR.

Even though the changes in phosphorylation state of NF-κB was undetectable in this experiment, the western blots results showed that in each group of different treatments, phosphorylation of JNK in iPFK2-KD adipocytes was drastically higher than that in iPFK2-Ctrl cells. The phosphorylation of JNK was quantified by densitometry and normalized by GAPDH. This confirmed that PFKFB3/iPFK2 contributes to anti-inflammatory response in adipocytes, and that a disruption of PFKFB3/iPFK2 could lead to inflammatory response through activation of JNK. When iPFK2-Ctrl was treated with metformin, phosphorylation of JNK decreased compared to PBS control with or without LPS stimulation. However, this improvement in JNK phosphorylation was not present in iPFK2-KD adipocytes (Figure 9). Thus, PFKFB3/iPFK2 was essential in the action of metformin in inhibiting the activation of JNK pathway.

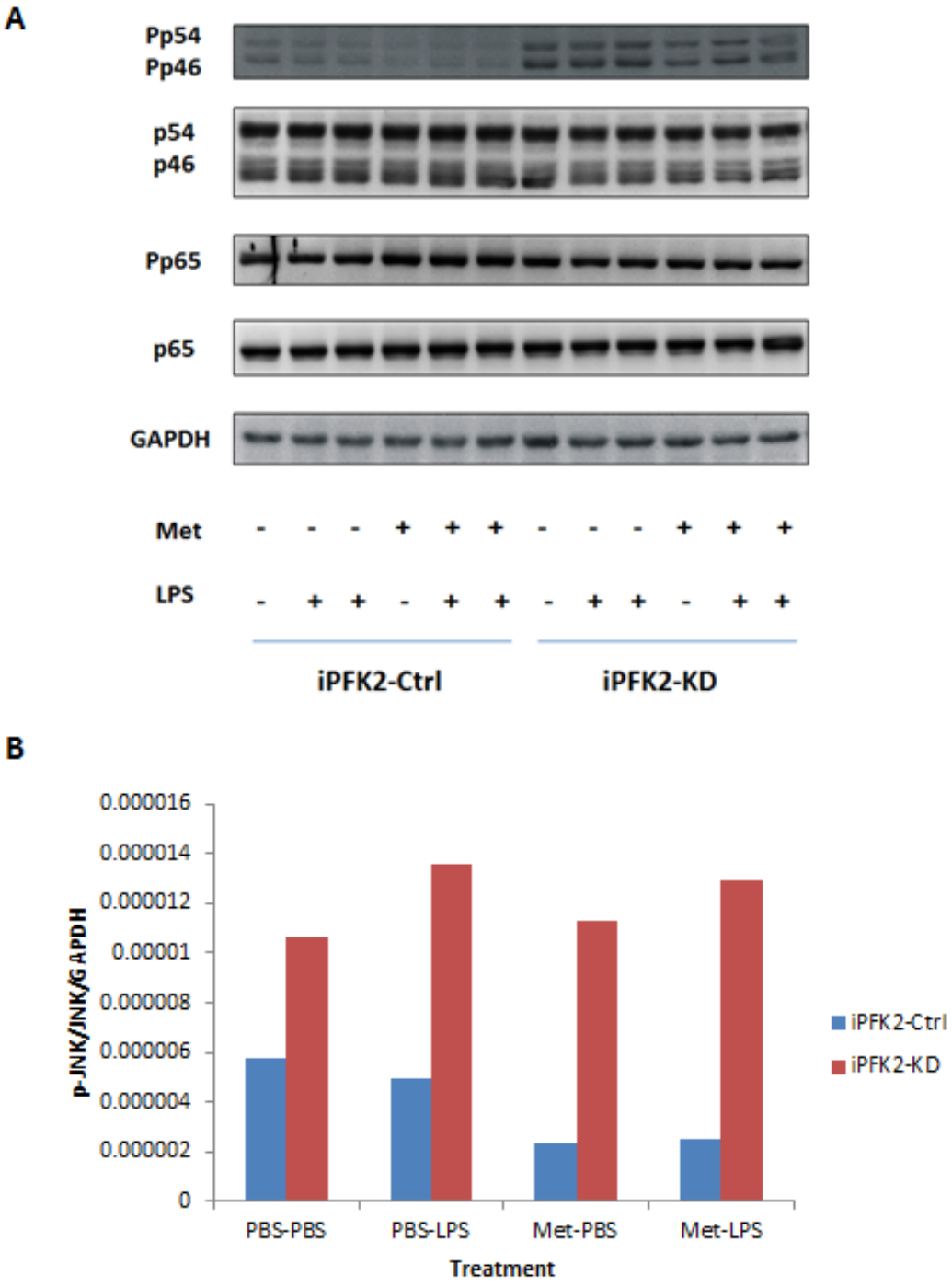

Figure 9. Anti-inflammatory effect of metformin on iPFK2-Ctrl and iPFK2-KD cells.

4.1.5 A disruption of PFKFB3/iPFK2 in 3T3-L1 adipocytes could impair effect of metformin treatment on expression of cytokines

The harvest and analysis of the RNA samples were described in above section. Data are presented as mean ± SE (n = 3). * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ for comparison of effect of metformin treatment within isogenic adipocytes, and † $p<0.05$, †† $p<0.01$ and ††† $p<0.001$ for comparison between iPFK2-Ctrl and iPFK2-KD adipocytes with same treatment.

When both iPFK2-Ctrl and iPFK2-KD adipocytes were treated with metformin for 24 hours with a concentration of 50 μM, with or without LPS induction, changes of expression levels of proinflammatory markers showed similar pattern. When iPFK2-Ctrl cells were treated with metformin without stimulation of LPS, expression levels of TNF-α, IL-1β, MCP-1 and TLR-4 significantly decreased. When induced with LPS, these proinflammatory markers' expression was enhanced in PBS-LPS group. IL-6 showed some trend of amelioration in this increase in expression levels, but this improvement was not significant. On the contrast, when iPFK2-KD adipocytes were treated with metformin without stimulation of LPS, relative mRNA expression levels of TNF-α, IL-6, MCP-1 and TLR-4 significantly increased. Under LPS stimulation, expressions of proinflammatory markers increased as expected. Interestingly, metformin treatment not only did not show improvement in this enhanced expression, but appeared to exacerbate them. In iPFK2-KD cells stimulated with LPS, metformin treatment increased MCP-1 expression with a significant p-value of 0.0159, and increased TLR-4 expression with a p-value of 0.0784 compared with PBS-LPS group (Figure 10).

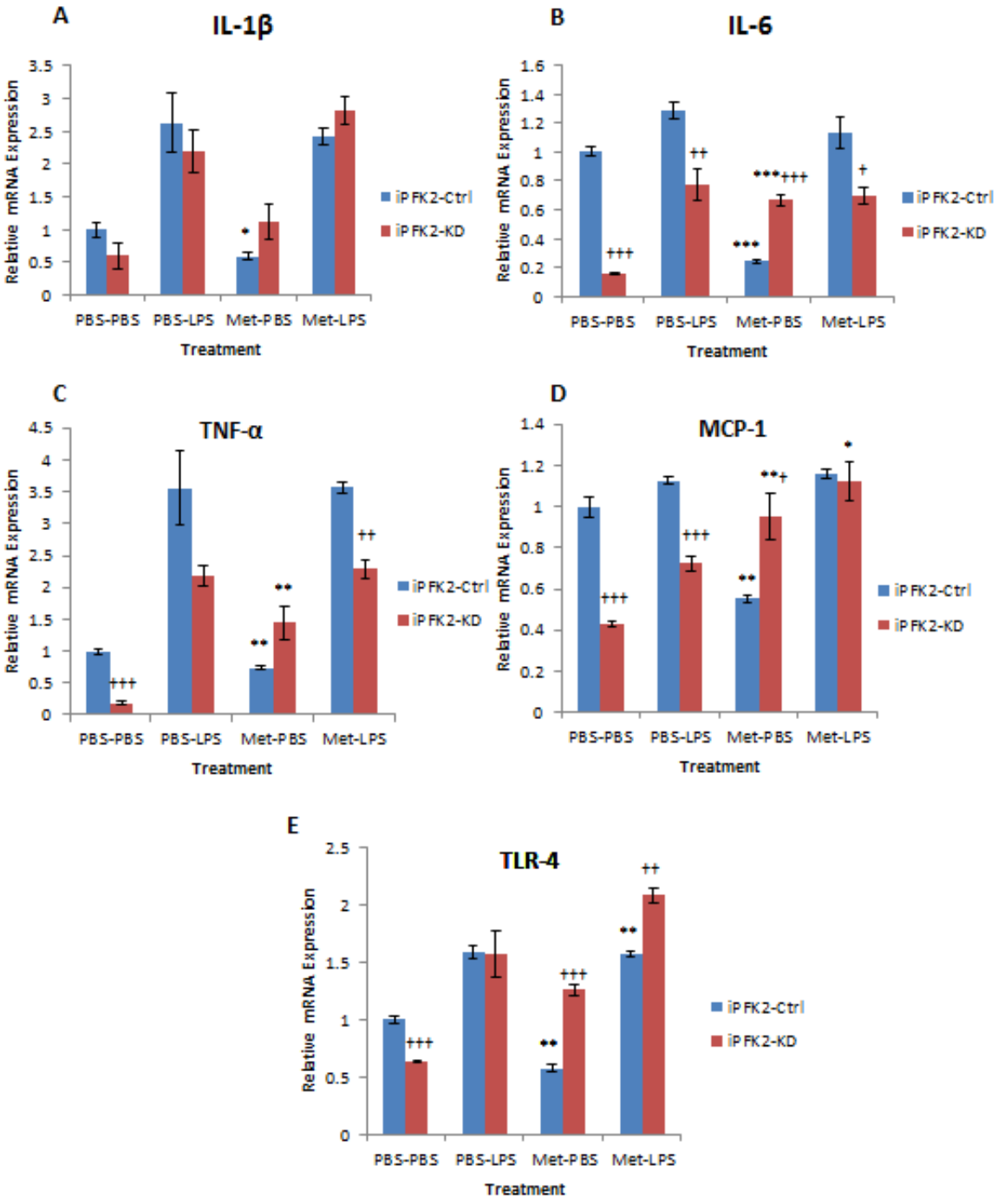

Figure 10. Effect of metformin on proinflammatory markers expression of iPFK2-Ctrl and iPFK2-KD cells.

4.1.6 Knockdown of PFKFB3/iPFK2 in 3T3-L1 adipocytes impaired the insulin-sensitizing effect of metformin

As stated previously, both iPFK2-Ctrl and iPFK2-KD adipocytes were treated with 50 μM metformin for 24 hours. Prior to harvest, insulin signaling response was explored with or without addition of insulin 30 minutes before harvest of protein samples. Changes in levels and phosphorylation states of Akt were used to observe insulin signaling response, quantified by ratio of p-Akt to total Akt, normalized by GAPDH.

In every treatment group in this study, iPFK2-KD adipocytes displayed a marked decrease of phosphorylation of Akt compared to iPFK2-Ctrl cells. iPFK2-Ctrl adipocytes exhibited a obvious increase in insulin-stimulated Akt phosphorylation in metformin treatment groups (Met-PBS and Met-Ins) compared to PBS control (PBS-PBS and PBS-Ins respectively), indicating the insulin-sensitizing effect of metformin in adipocytes. However, when PFKFB3/iPFK2 was disrupted, this beneficial effect of metformin was impaired. In iPFK2-KD cells, metformin did not improve insulin signaling in basal conditions in absence of insulin, and furthermore, showed a tendency to inhibit insulin signaling in insulin-stimulated condition (Figure 11).

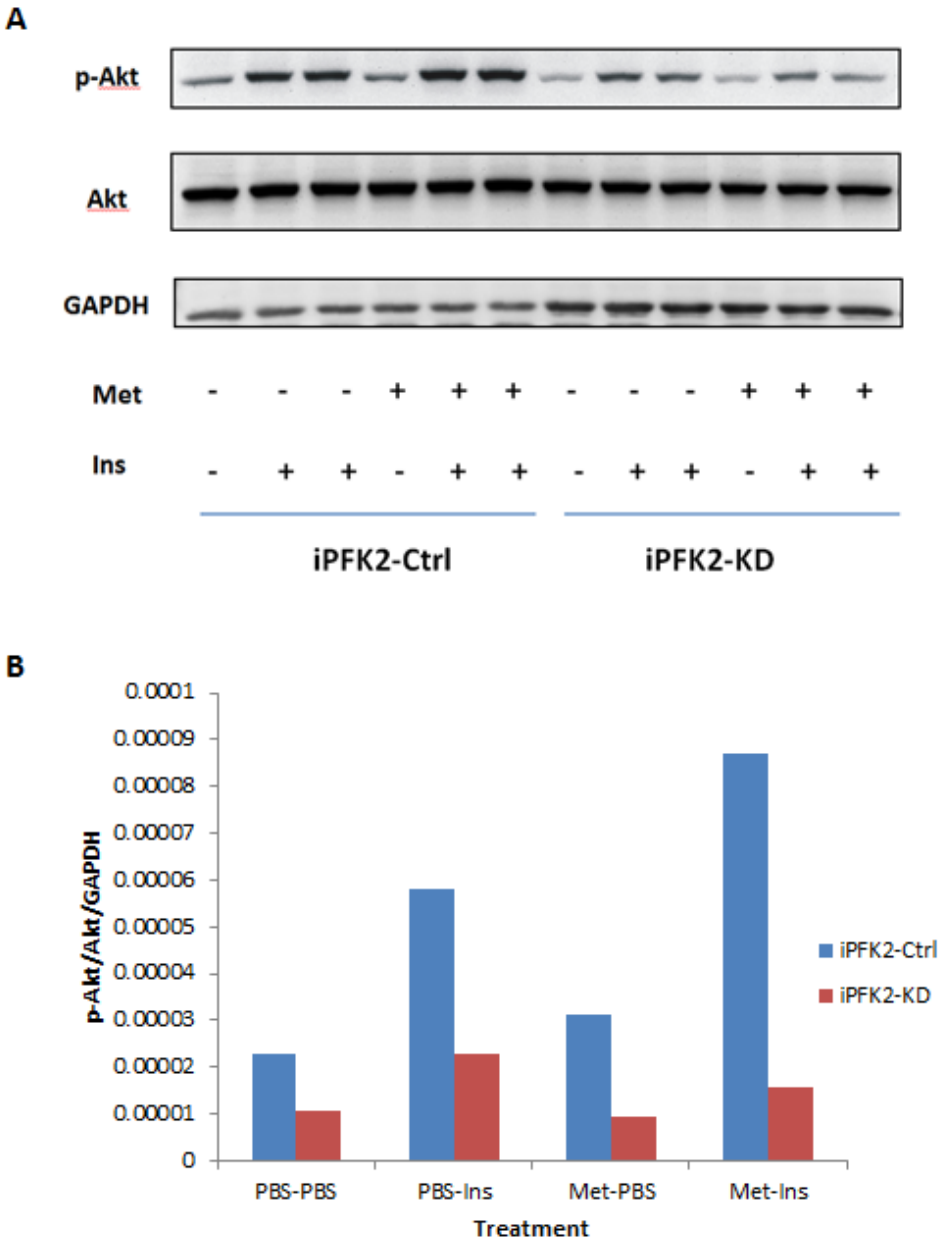

Figure 11. Effects of metformin on insulin signaling of iPFK2-Ctrl and iPFK2-KD cells.

4.1.7 AMPK phosphorylation may be a potential pathway involvement of PFKFB3/iPFK2 in metformin treatment in adipocytes

Since the data above suggested that PFKFB3/iPFK2 was involved in the metformin action in anti-inflammatory and insulin-sensitizing aspects, we wanted to explore the possible mechanism underlying this involvement. Because of the highly activated AMPK pathway in metformin action was established in liver and muscle in many studies, and that AMPK is the upstream of PFKFB3 in its modulation [67], we hypothesized that in adipocytes, the involvement of PFKFB3/iPFK2 was also through the phosphorylation state of AMPK. Therefore, p-AMPK and AMPK levels were measured in our study.

In dose and time course effect of metformin, PFKFB3/iPFK2 levels appeared to increase with metformin treatment. However, phosphorylation of AMPK under LPS stimulation did not alter much with different doses and time course of metformin treatment, with metformin at 50 μM and 6 h being slightly higher than other concentration and time courses (Figure 12).

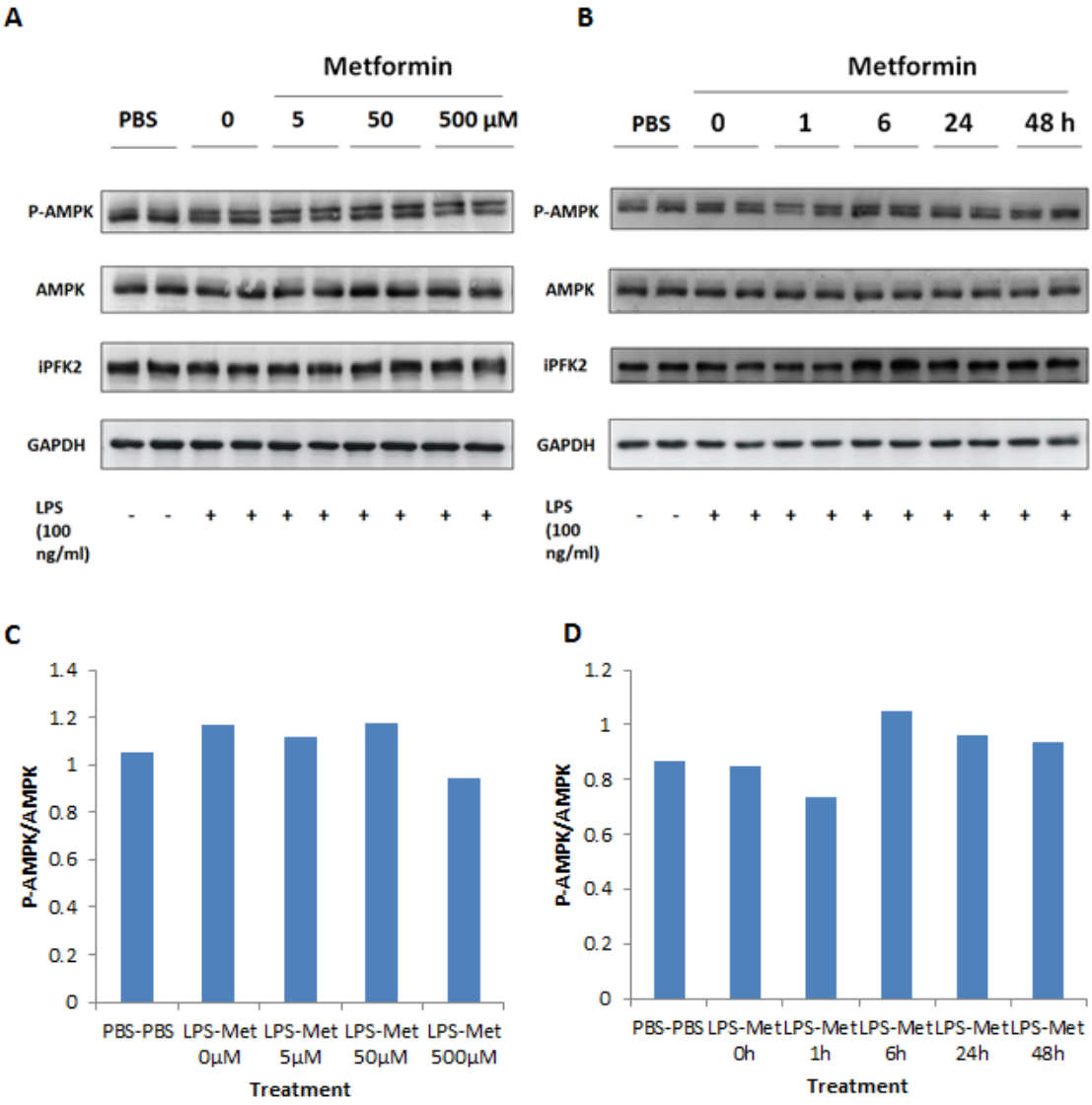

Figure 12. Dose-dependent and time-dependent effect of metformin on PFKFB3/iPFK2 and phosphorylation of AMPK.

When comparing the AMPK phosphorylation states in iPFK2-Ctrl and iPFK2-KD adipocytes under metformin treatment, the phosphorylation of AMPK on iPFK2-Ctrl cells was markedly increased with metformin treatment, but was not altered in iPFK2-KD cells (Figure 13). In iPFK2-Ctrl cells, AMPK was phosphorylated for metformin to take action; but when in PFKFB3/iPFK2-disrupted conditions, AMPK was not activated. This suggested that AMPK phosphorylation might be a potential pathway of how PFKFB3/iPFK2 was involved in metformin treatment in adipocytes.

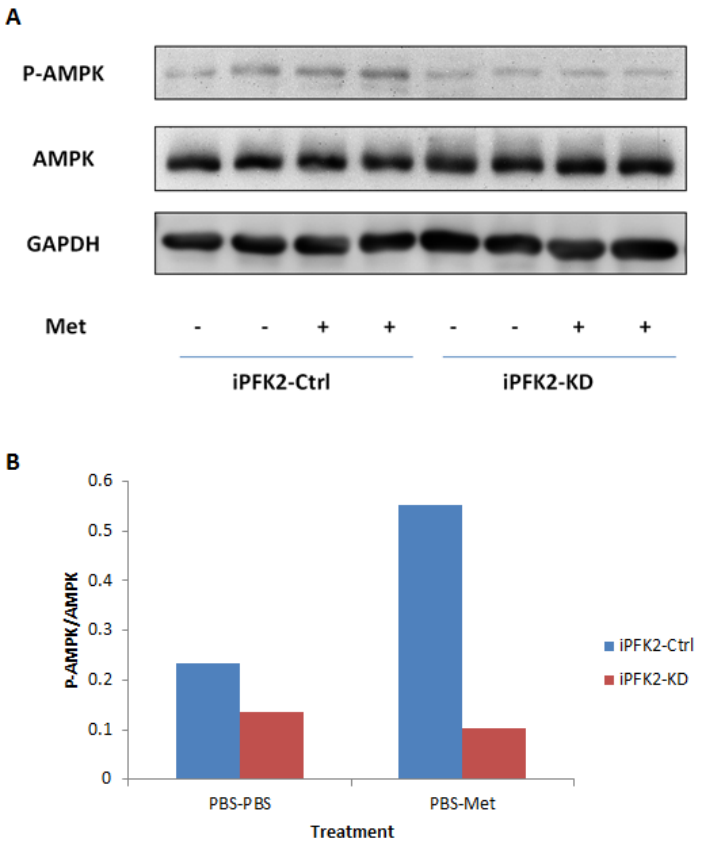

Figure 13. Metformin effect on AMPK phosphorylation on iPFK2-Ctrl and iPFK2-KD cells.

4.2 Temporal effect of pioglitazone on 3T3-L1 adipocytes

4.2.1 3T3-L1 adipocytes displayed circadian rhythms

In the first set of experiment, we first wanted to confirm the existence of circadian rhythm within 3T3-L1 adipocytes by examining the expression of core clock genes and levels of clock proteins. The protein levels of CLOCK, BMAL1 and PFKFB3/iPFK2 were measured by western blot of the protein samples that we harvested at different circadian times. RT-PCR was utilized to investigate the quantitative expression levels of core clock genes, including Clock, Bmal1, Per1, Per2, as well as our interested gene PFKFB3.

After normalizing with GAPDH, both CLOCK and BMAL1 exhibited oscillations in their protein levels, with ZT1 and ZT19 being higher, and ZT7 and ZT13 being lower. The magnitude of oscillation in CLOCK was smaller to that in BMAL1. We also measured the protein levels of PFKFB3/iPFK2, which did not display obvious changes at different circadian times (Figure 14).

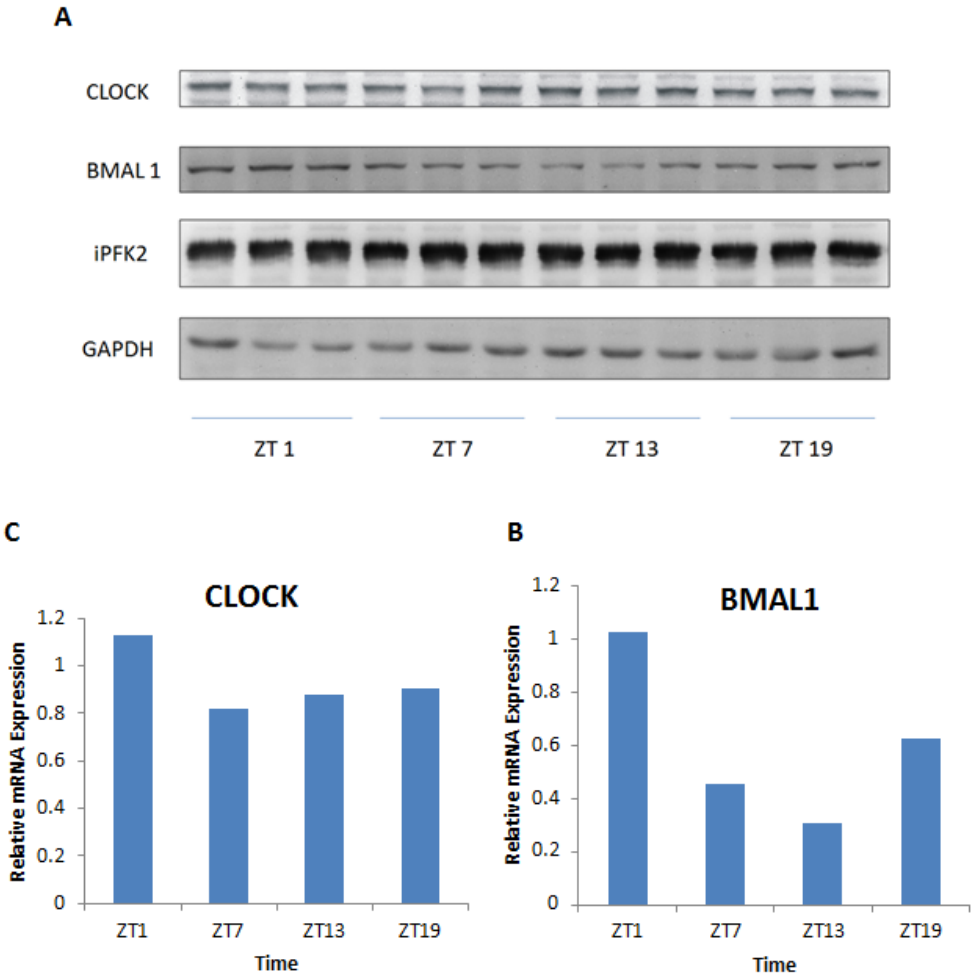

Figure 14. Levels of core clock proteins through different circadian times.

Relative mRNA expression of core clock genes was shown in Figure 15, with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ for significance level in comparison with ZT1. Clock and Bmal1 displayed similar circadian oscillation, evidenced by a decreased expression at ZT13 and markedly increased expression at ZT19 (Figure 15). Per 1 exhibited some oscillation, but expression levels at ZT7, ZT13 and ZT19 were not

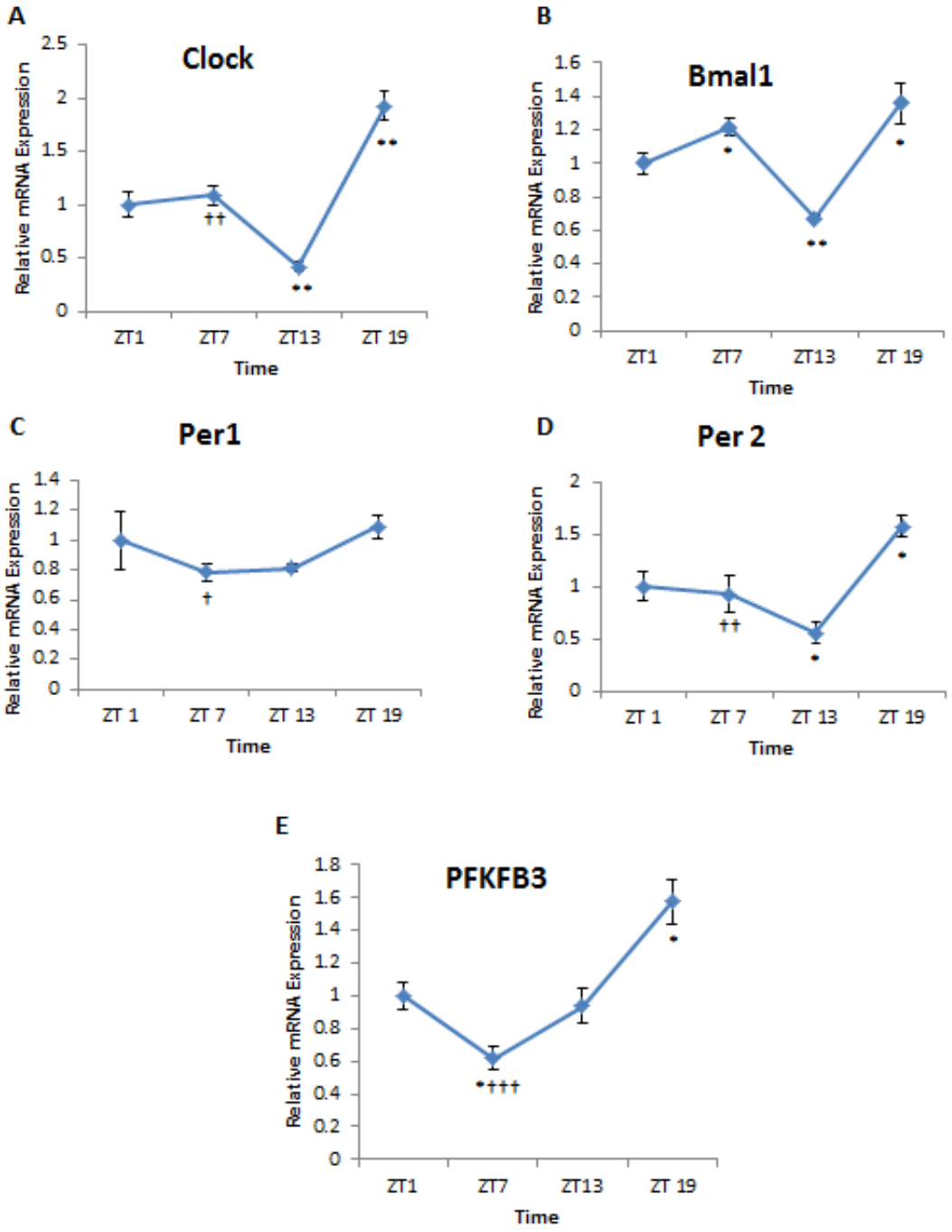

Figure 15. Expression of core clock genes at different circadian times.

statistically significant different from ZT1. Expression of Per2 was rhythmic with a similar patter to Clock, except that the magnitude of amplification was smaller. To our surprise, Clock and Bmal1 circadian oscillation were not obviously antiphase to the rhythm exhibited by Per1 and Per2. PFKFB3, our interested gene, also displayed noticeable rhythmic oscillation in its expression, with its peak expression at ZT19 and nadir expression at ZT7.

Since all of the genes examined displayed a peak expression at ZT19, ZT19 was selected as one treatment time point for the following study. To ensure a 12-hour treatment window, ZT7, as another treatment time point. The relative expression of the majority of our genes were significantly different at ZT7 and ZT19, indicated by † p-value<0.05, †† p-value<0.01 and ††† p-value<0.001.

4.2.2 Treating 3T3-L1 adipocytes with pioglitazone at different circadian times may have different inflammatory response

After proving the circadian oscillation in wild-type 3T3-L1 adipocytes among core circadian components, our aim is to explore that whether treating cells with pioglitazone at different circadian times could lead to differential results. As stated previously, ZT7 and ZT19 were designated as two circadian time points for pioglitazone treatment based on our results in the last section. After synchronization, pioglitazone was treated to 3T3-L1 adipocytes for 48 hours. LPS was added 30 minutes to induce inflammatory response.

As shown in Figure 16, without addition of any treatment to adipocytes, DMSO-PBS group exhibited lessened inflammatory state at ZT19, indicated by lower levels of

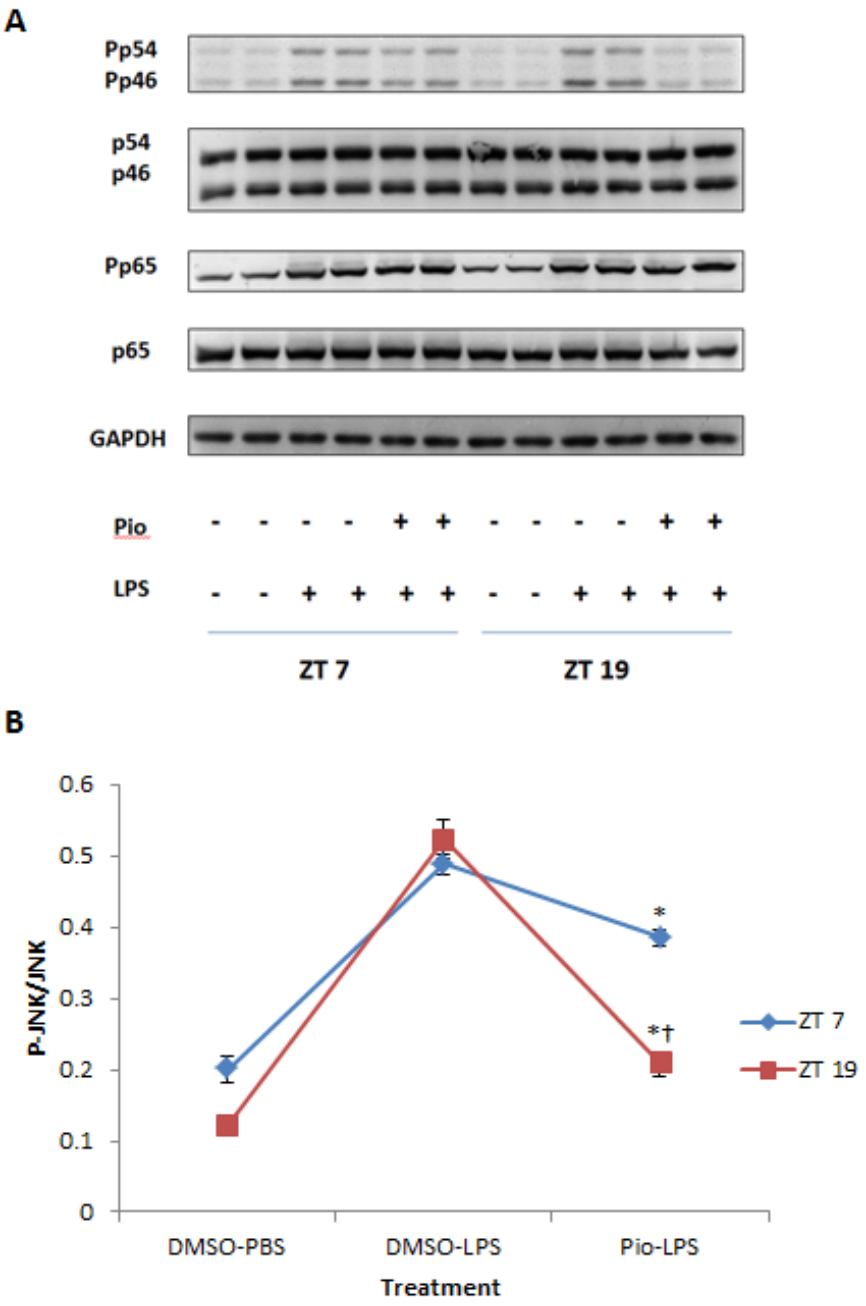

Figure 16. Anti-inflammatory effect of pioglitazone on adipocytes at ZT7 and ZT19.

JNK phosphorylation. When cells were stimulated with LPS, p-JNK increased as anticipated, to an extent that was similar for circadian times, suggesting that LPS induced a stronger inflammatory response at ZT19. Pioglitazone treatment generated differential anti-inflammatory effects at two time points, indicated by remarkably lowered phosphorylation level of JNK at ZT19, when inflammatory response was stronger. This effect was not as strong at ZT7. There was no marked difference in changes of p-NF-κB (Figure 16).

4.2.3 Treating 3T3-L1 adipocytes with pioglitazone at different circadian times did not alter insulin-sensitizing effect

To investigate the temporal effect of pioglitazone in insulin sensitivity, similarly, after synchronization, pioglitazone was treated to 3T3-L1 adipocytes at ZT7 and ZT19 for 48 hours. Insulin was added 30 minutes prior to harvest to stimulate insulin signaling response. Proteins samples were analyzed by western blot.

Adipocytes displayed different levels of Akt phosphorylation at basal conditions (DMSO-PBS), with higher p-Akt levels at ZT19. Under insulin stimulation, p-Akt increased at ZT7 and ZT19 to an extent that was similar for both circadian times, indicating that adipocytes were more responsible to insulin at ZT7. However, similar phosphorylation levels of Akt suggested that the addition of pioglitazone did not markedly increase insulin signaling at ZT7 and ZT19 (Figure 17).

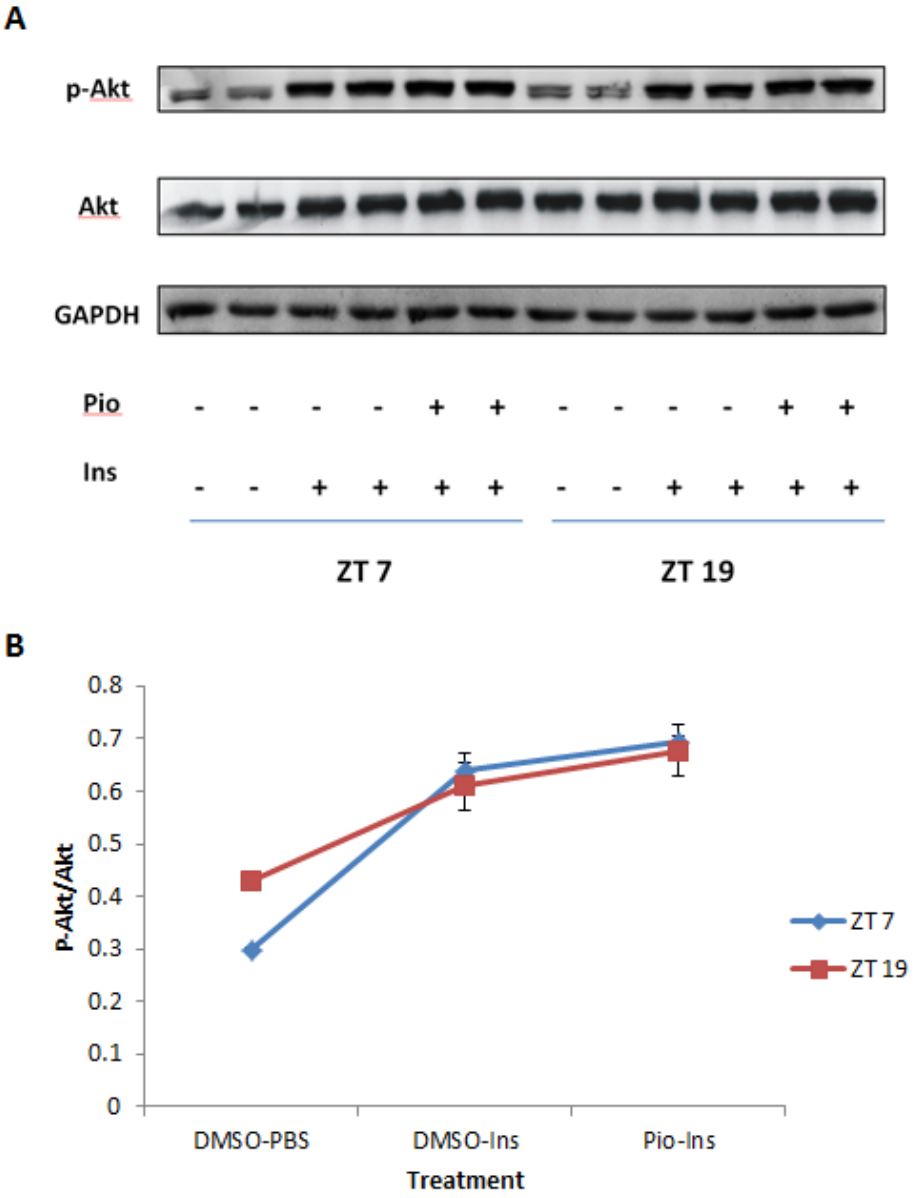

Figure 17. Insulin-sensitizing effect of pioglitazone on adipocytes at ZT7 and ZT19.

5. SUMMARY AND CONCLUSIONS

5.1 Summary

5.1.1 Involvement of PFKFB3/iPFK2 in beneficial effects of metformin on adipocytes

Treating 3T3-L1 with metformin at 50 μM for 24 hours could ameliorate the inflammatory response to LPS induction, evidenced by phosphorylation of JNK and NF-κB, and expression of proinflammatory cytokines TNF-α and IL-1β.

In western blot analysis for protein samples and RT-PCR for RNA samples, compared with that in iPFK2-Ctrl adipocytes, metformin did not exhibit anti-inflammatory response when PFKFB3/iPFK2 was knockdown. In iPFK2-KD cells, phosphorylation state of JNK was not improved with metformin group, and relative mRNA expression of TNF-α, IL-1β, IL-6, MCP-1 and TLR-4 was not reduced under metformin treatment. These data suggested that disruption of PFKFB3/iPFK2 weakened the anti-inflammatory effect of metformin in adipocytes. In PFKFB3/iPFK2-Control (iPFK2-Ctrl) adipocytes, metformin treatment was shown to have significant insulin-sensitizing effect, evidenced by an increase in p-Akt/Akt signaling. In contrast, insulin sensitivity was not improved, but evidently impaired under metformin treatment on iPFK2-KD adipocytes.

In wild-type 3T3-L1 adipocytes, PFKFB3 was upregulated with metformin treatment under LPS stimulation, shown by a marked increase in relative mRNA expression, suggesting its potential involvement in metformin action. Meanwhile, the phosphorylation of AMPK, the upstream of PFKFB3, in iPFK2-Ctrl adipocytes was

increased with metformin treatment, but did not alter much in iPFK2-KD cells. This indicated the AMPK pathway as the mechanism of involvement of PFKFB3/iPFK2 in metformin treatment.

5.1.2 Temporal effects of pioglitazone on adipocytes

In 3T3-L1 adipocytes, circadian rhythm was observed in both protein samples and RNA samples harvested at different circadian times. Temporal variation for protein levels was displayed in CLOCK and BMAL1 with similar patterns. Relative mRNA expression of several clock genes Clock, Bmal1, Per1 and Per2 exhibited oscillation. PFKFB3 also showed a rhythmic expression through different times.

Under LPS stimulation, 3T3-L1 adipocytes experienced more inflammatory response at ZT19. Pioglitazone treatment at ZT 19 was more effective in reducing inflammatory response through dephosphorylation of JNK, but changes in phosphorylation state of NF-κB were now seen. Even though adipocytes displayed differences in insulin signaling in basal conditions, pioglitazone treatment did not show significant insulin-sensitizing effect, and no notable differences were seen between pioglitazone treatments at different circadian times.

5.2 Conclusions

Taken together, the results generated from this study support the hypothesis of the beneficial effects of metformin in 3T3-L1 adipocytes and the critical involvement of PFKFB3/iPFK2. Metformin treatment on 3T3-L1 adipocytes could increase insulin sensitivity and ameliorate inflammatory response through dephosphorylation of JNK and

inhibition of proinflammatory cytokine expression. A disruption of PFKFB3/iPFK2 in adipocytes impairs the insulin-sensitizing and exacerbates anti-inflammatory effect of metformin. This involvement of PFKFB3/iPFK2 in metformin action is possibly through the AMPK pathway.

3T3-L1 adipocytes display an internal oscillation, evidenced by temporal variations in levels of core clock proteins and expression of core clock genes. Furthermore, 3T3-L1 adipocytes responded circadian time-differently when stimulated with LPS and insulin. Anti-inflammatory effect of pioglitazone at ZT19 is more effective than that at ZT7, while no significant insulin-sensitizing effect was seen in both time points.

5.3 Future experiments

According to the generated data, there are some suggestions for future experiments in the following:

1. Using iPFK2-Ctrl and iPFK2-KD mice models to investigate the involvement of PFKFB3/iPFK2 in metformin treatment in high-fat diet mice, in regards to inflammatory and insulin-sensitizing effects, and activation of AMPK
2. Since PFKFB3/iPFK2 also displays circadian oscillation in 3T3-L1 adipocytes, iPFK2-Ctrl and iPFK2-KD cell lines could be utilized to investigate temporal drug effects and the involvement of PFKFB3/iPFK2

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